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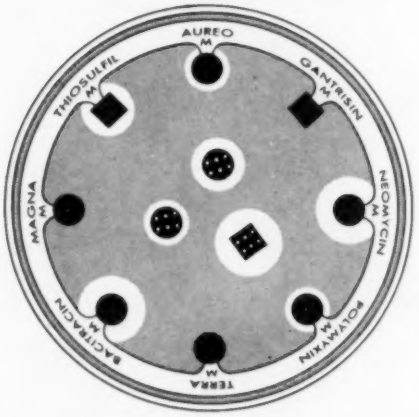

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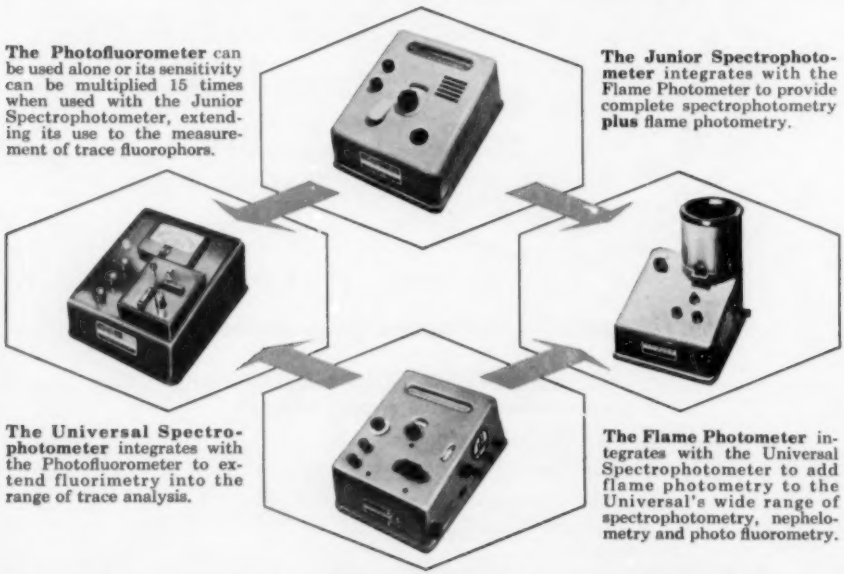

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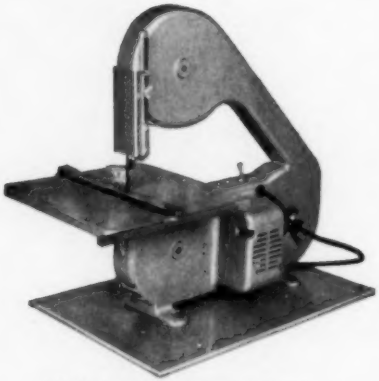

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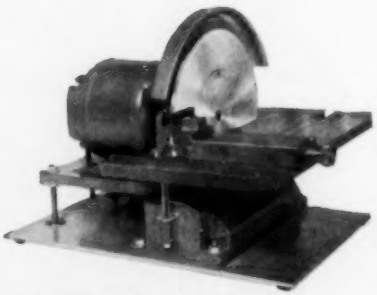

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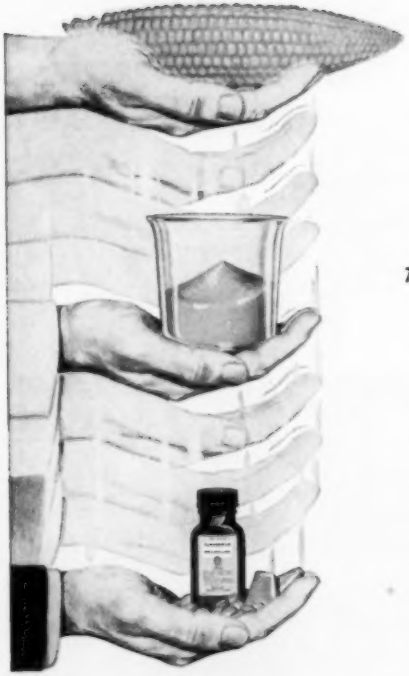

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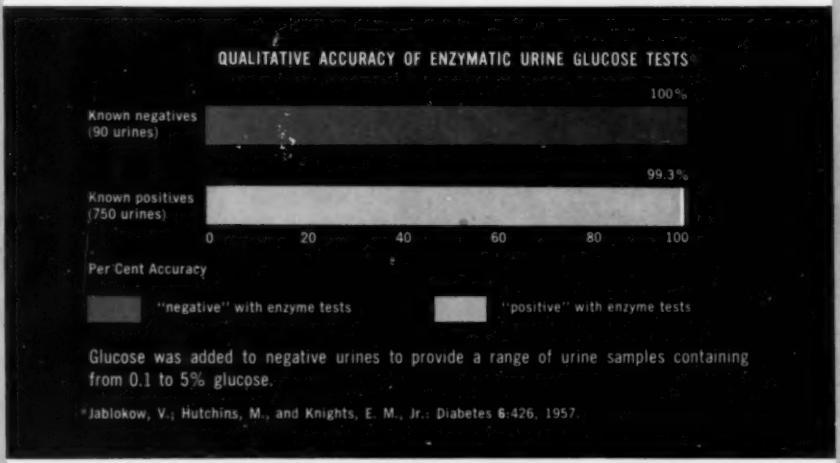

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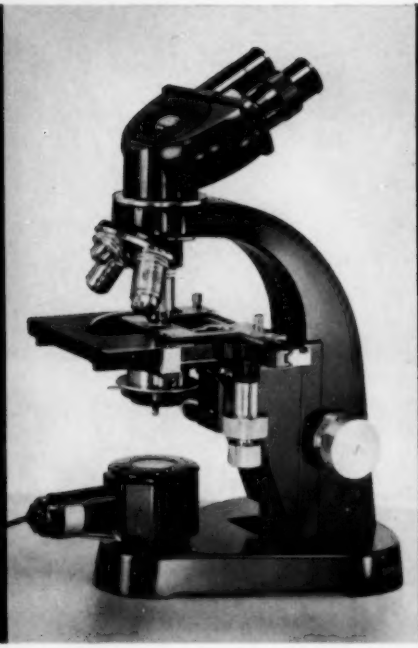

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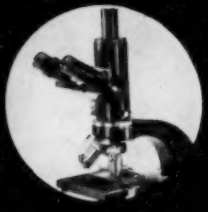

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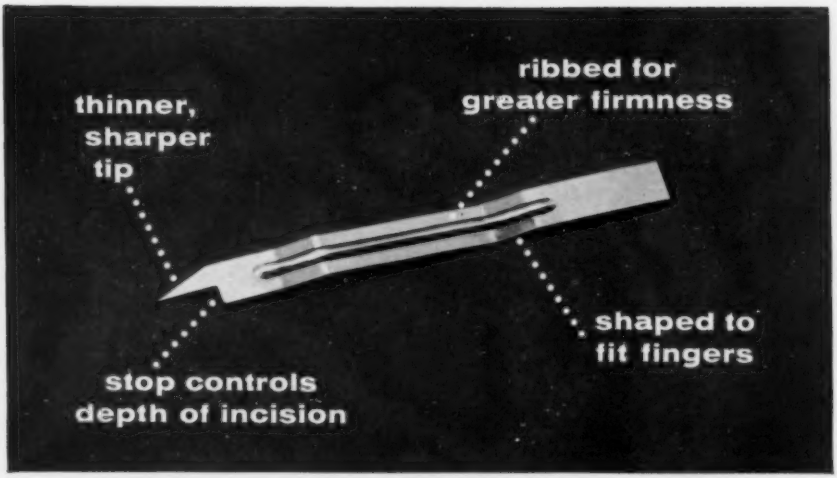

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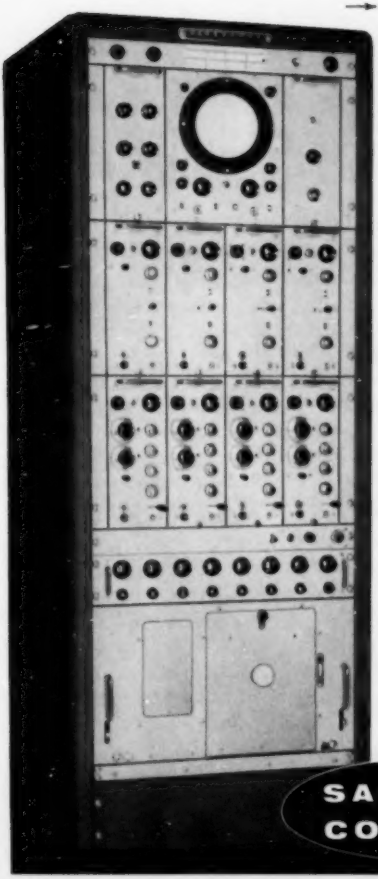

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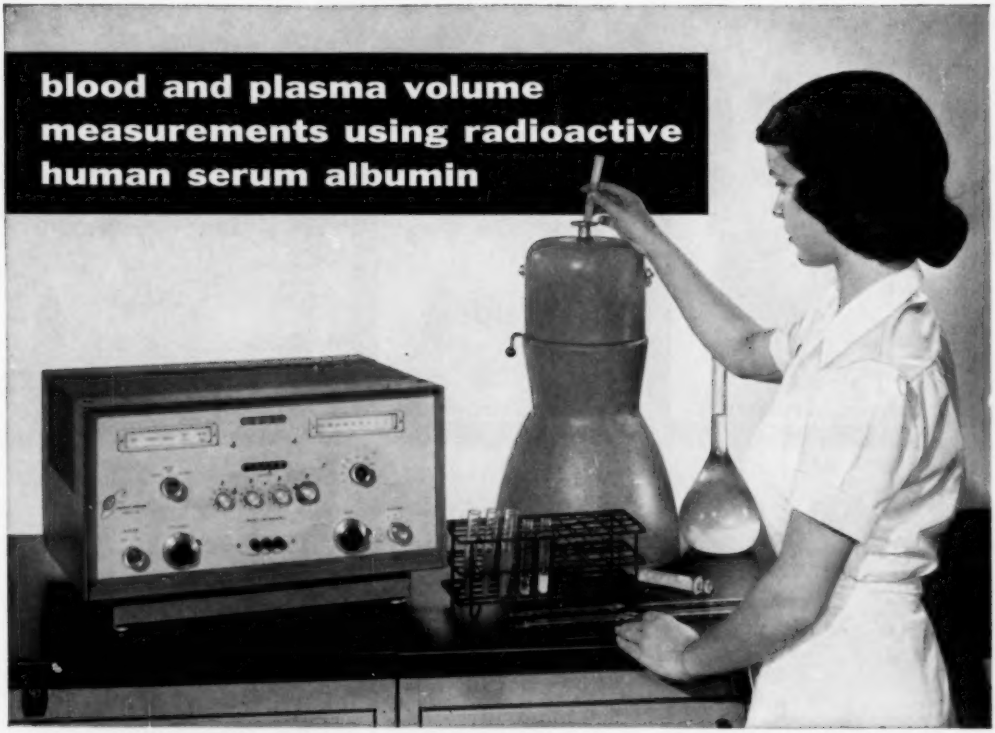

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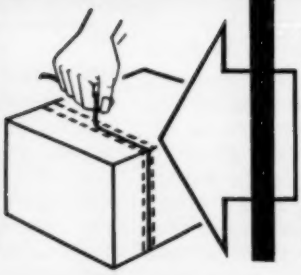

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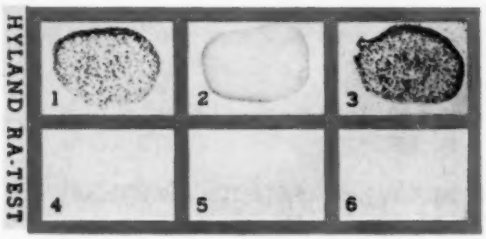

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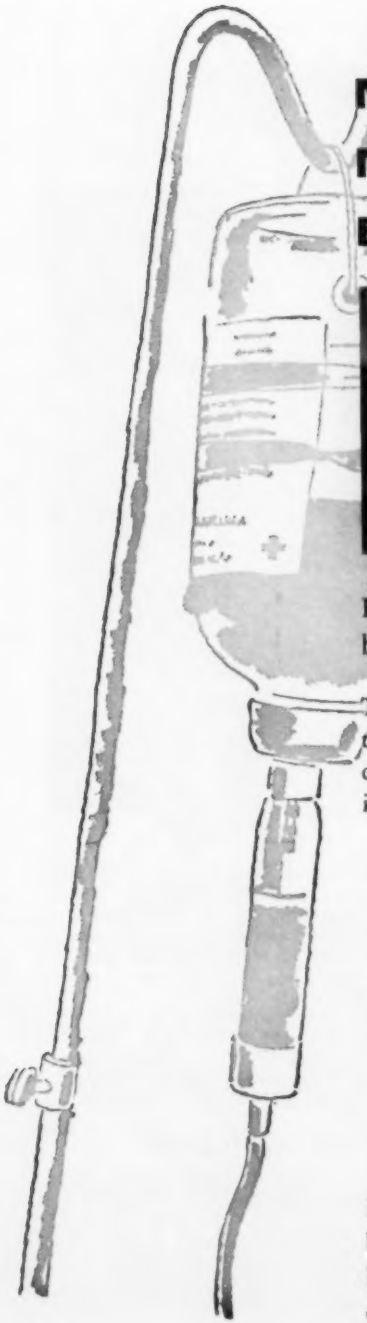

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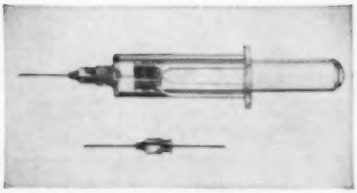

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CLINICAL AND EXPERIMENTAL

IMPAIRMENT OF HEPATIC BROMSULPHALEIN CLEARANCE BY TWO 17-SUBSTITUTED TESTOSTERONES

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BETHESDA, MD.

THE ability of the liver to clear the blood of the dye, Bromsulphalein (BSP), has proved to be an invaluable diagnostic and investigative tool in studies of hepatic function. However, since neither the mechanisms of BSP uptake and excretion nor the precise hepatic function which this uptake measures is adequately understood, the association of high BSP retention with hepatocellular dysfunction remains primarily an empirical one. It is the purpose of this communication to describe what appears to be an exception to this relationship: elevated BSP retention, in the absence of other laboratory or clinical evidence of liver dysfunction has been consistently produced by two commonly used 17-substituted testosterones.

MATERIALS AND METHODS

Patients reported in this study were hospitalized on the service of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health. All were treated with an anabolic steroid because of some indication related to their primary disorders. In conjunction with this therapy, routine analyses of liver function were performed before and during steroid therapy. These liver function studies consisted of routine determination of 45-minute BSP retention (5 mg. BSP per kilogram body weight), serum bilirubin, cephalin-cholesterol flocculation, thymol turbidity, total cholesterol, total protein, A/G ratio, alkaline phosphatase, and in some instances, prothrombin time. Four young adult, normal control subjects were also studied while receiving one or more of the

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TABLE I. FORTY-FIVE-MINUTE BSP RETENTION VALUES AND BSP CLEARANCE RATES (CR) IN PER CENT PER MINUTE IN 11 SUBJECTS BEFORE AND DURING TREATMENT WITH VARIOUS TESTOSTERONES*

			METHYLTESTOSTERONE					TESTOSTERONE PROPIONATE					NORETHANDROLONE				
				CONTROL		TREATMENT			CONTROL		TREATMENT			CONTROL		TREATMENT	
PATIENT	DIAGNOSIS	AGE	DOSE (MG./DAY)	45′ RET. (%)	CR	45′ RET. (%)	CR	DOSE (MG./WK.)	45′ RET. (%)	CR	45′ RET. (%)	CR	DOSE (MG./DAY)	45′ RET. (%)	CR	45′ RET. (%)	CR
1. C. O.	Osteoporosis	67											100	5	3.2	27	0.3
2. G. S.	Osteoporosis	52	100	4		17							50	4		21	
3. E. S.	Osteogenesis imperfecta	8											20	4		12	
4. J. J.	Dermatomyositis	54											80	2		18	
5. G. A.	Osteoporosis rheum. arth.	66	100	2		34							100	2		31	
6. W. F.	Normal	20											100	3	4.3	27	1.2
7. M. H.	Normal	22	100	3	4.7	25	0.5										
8. O. B.	Osteoporosis rheum. arth.	59											100	4		25	
9. J. McM.	Osteoporosis rheum. arth.	56	100	4		32		150	4		3		100	3		18	
10. L. C.	Normal	20						150†	2	4.5	1	4.7	100	2	4.5	22	0.8
L. C.													20	2	4.5	19	1.4
11. W. Y.	Normal	22	100	3	4.2	23	1.5	150†	3	4.8	3	4.7	30	3	4.8	24	1.2

*Doses of methyl testosterone and norethandrolone are given as milligrams per day, testosterone propionate as milligrams per week (in 3 injections per week). Values represent largely single determinations in Cases 1 to 4, means of from 2 to 4 determinations in Cases 5 to 7 and means of more than 4 determinations in Cases 8 to 11.

†Dose increased to 100 mg. per day, intramuscularly, for 3 days preceding the final test.

anabolic steroids; in these subjects in addition to the above liver function studies, serum BSP clearance rates were measured. These latter were performed by giving the dye intravenously in a dose of 5 to 6 mg. per kilogram, and taking serial blood samples at intervals of 5 to 10 minutes, for up to 90 minutes, through an indwelling needle in the opposite arm. All such studies were performed fasting, under basal conditions. The clearance rate is taken as −100 times the slope (k) of the final portion of the curve of the logarithm of serum BSP concentration against time. BSP values were determined by the method of Gaebler.[1]

The agents studied were testosterone propionate administered intramuscularly, methyltestosterone, and norethandrolone (17-ethyl-19-nortestosterone, Nilevar) administered orally (not sublingually).

RESULTS

Table I lists the patients, their diagnoses, and mean 45-minute BSP retention values before and during therapy with the various steroids. BSP clearance values (CR) are also listed for the 4 normal control subjects and for the one patient in whom this determination was performed. Both 17-substituted testosterones (methyltestosterone and norethandrolone) in doses ranging from 20 to 100 mg. per day, produced large elevations of 45-minute BSP retention in all patients so treated. Even at the lower doses, retention and clearance values were plainly abnormal, and, indeed, there is little apparent relationship between the size of the dose and the extent of the change. In none of these patients were any symptoms or signs of hepatic dysfunction noted: there was neither clinical jaundice nor significant change in the serum bilirubin, and no changes occurred in cephalin-cholesterol flocculation, thymol turbidity, alkaline phosphatase, total protein, or prothrombin time. There was an inconstant change in the total cholesterol in the subjects receiving the 17-substituted testosterones: most patients showed a slight to moderate elevation during treatment; some showed no appreciable change; and one showed a marked fall, followed by gradual restoration to pretreatment values after 2 months of therapy. (Both elevations and depressions of cholesterol have been described previously with methyltestosterone.[2-4])

Three patients studied with testosterone propionate showed no changes in BSP retention on a dose of 50 mg., intramuscularly, given 3 times weekly, continued for as long as 2 months. In two of the subjects, after receiving this dose for a minimum of one month, the drug was increased to 100 mg. per day, intramuscularly, for the 3 days preceding the final study. This dose is equivalent anabolically to 300 to 400 mg. of the 17-substituted compounds, and yet, even under these circumstances, no apparent effect on BSP clearance was observed. Similarly, no changes were noted in serum cholesterol or in other liver function studies in the patients receiving testosterone propionate.

BSP clearance values showed changes comparable in extent to those noted in 45-minute dye retention; in all cases treated with the 17-substituted testosterones, values dropped from the mean pretreatment value for the normal control subjects of 4.8 ± 0.6 per cent per minute to a mean of 1.1 per cent per minute. Fig. 1 shows two typical BSP disappearance curves and illustrates clearly the marked effect upon BSP removal from the blood produced by

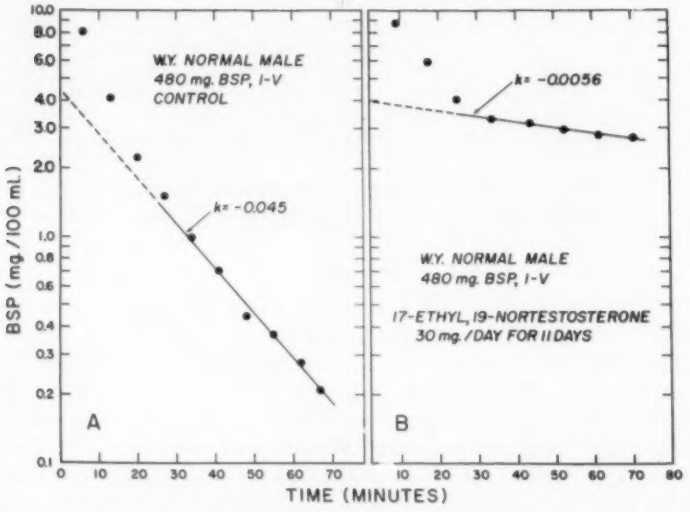

Fig. 1.—Hemilogarithmic plot of serum BSP concentration against time in Patient W. Y. *A*, Control; *B*, after 11 days treatment with norethandrolone, 30 mg. per day. The dose of the dye was the same in both cases (5.5 mg. per kilogram).

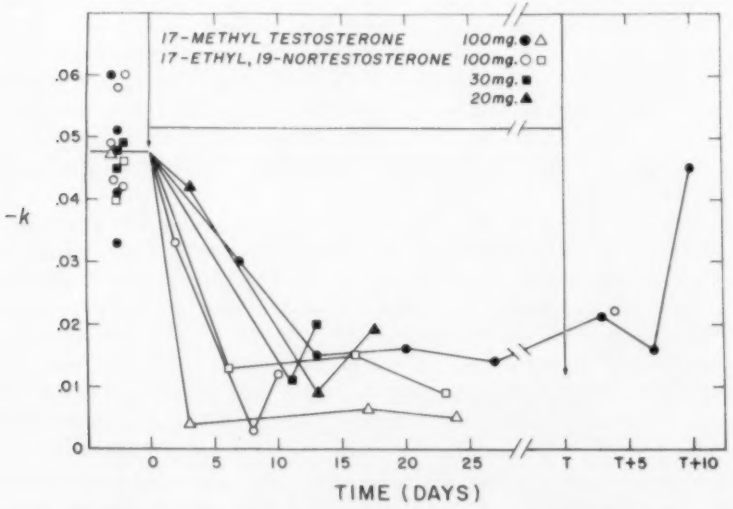

Fig. 2.—Effect of 17-methyltestosterone and 17-ethyl-19-nortestosterone on serum BSP clearance rates. Control points are grouped to the left of the time scale. T represents the date of termination of therapy. Additional posttreatment clearances, performed more than 10 days after stopping therapy, have all been within the normal range and are not included on this graph.

these steroids. The time course of this phenomenon is shown in Fig. 2. Maximum depression was obtained in all cases within 7 to 10 days after starting treatment, and conversely normal values were restored within 7 to 10 days after stopping therapy. Again, no such changes were observed with testosterone propionate.

Analysis of the BSP curves revealed two decreasing exponential segments. The clearance rates are based on the slope of the final segment which varied somewhat from person to person, in the same subject from study to study, and which was markedly altered by the 17-substituted testosterones. The half-time of the initial segment, however, remained sensibly constant for each individual throughout the entire series of studies. The values for this half-time ranged from 3.5 to 6 minutes in the 5 patients tested. Whereas such rates are clearly so rapid that small changes would be altogether undetectable with the methods employed, it is reasonably certain that there were at least no gross changes in the mechanism responsible for the rapid initial fall in serum dye concentration.

DISCUSSION

Although the number of studies is small, it seems reasonably well established that the observed effect on BSP removal is peculiar to the two 17-substituted testosterones and is not exhibited by testosterone propionate even in equimolecular dosage. This point is seen clearly in patients J. McM., L. C., and W. Y., all of whom were studied while receiving testosterone propionate as well as while on one or both of the 17-substituted compounds. All 3 subjects failed to demonstrate the defect with testosterone propionate but did so uniformly when receiving the 17-substituted compounds. It, therefore, seems reasonable to attribute the specific retention of BSP to the alkyl substitution on position 17.

The routine liver function studies performed, of course, do not measure a great many of the synthetic or excretory capacities of the liver, and, in the absence of such studies or of liver biopsies, no final statement can be made about the extent or significance of the observed effect. While this work was in preparation, a report has appeared by Kory and associates[5] also describing BSP retention in association with norethandrolone therapy. Six of their patients were subjected to liver biopsy, and the findings in all were normal. In the absence of evidence of liver dysfunction by any of the usual indices, it seems possible that such a readily reversible change in the handling of an unnaturally occurring substance does not represent liver cell damage, at least in the ordinary sense of the word.

The present studies offer no insight into the possible relationship of this regularly occurring effect to the rarely observed "methyltestosterone jaundice." However, years of clinical experience with methyltestosterone support the view that this agent is only rarely a hepatotoxin.

Serum BSP curves observed in these studies exhibited a form somewhat different from that described by Ingelfinger and associates.[6] These workers

described a simple exponential curve, and observed a "break" in the curve only in subjects with liver damage. As has been mentioned, a "break" was observed in all of the present subjects, including the normal controls. The most probable explanation for this difference is that the curves of Ingelfinger and associates were based upon serum BSP concentration taken only up to 30 minutes after injection. It is our experience that this period may not be long enough to observe a "break" in normal subjects, and that if the curve is followed for 60 to 70 minutes a slower segment will become apparent in all individuals. Accordingly, clearance rates derived from the present study, since they apply to the slow segment of the curves, are all considerably lower than those previously described.

The most plausible interpretation of biphasic curves of this sort, and the one which seems best to fit the known distribution of BSP,[7] postulates a large intrahepatic protein reservoir of dye-binding sites. The first segment would represent mixing within a large volume of such protein dye-binding sites and establishment of an equilibrium between intravascular protein-bound dye and hepatocellular-bound dye. The slower second segment would represent clearance of dye by the hepatic excretory mechanism. In the present instance, since the rate at which the injected dye was diluted within its "volume of distribution" did not change detectably with treatment, and since the rate of fall after equilibrium was markedly reduced, it seems probable that the observed defect involves the excretory mechanism. Indeed, the clearance rates in several treated subjects approximated that observed by Cohn and associates in hepatectomized dogs,[8] and in certain of the present studies easily detectable BSP concentrations were observed for as long as 6 hours after injection. The minute to minute disappearance of the dye from the blood in such studies, despite high serum concentrations, was less than 1 mg. per minute, a value clearly smaller than the normal upper limit of liver excretory capacity (as opposed to liver uptake) suggested by the studies of Combes and co-workers.[9]

It is not possible with the present data to exclude some reduction in total hepatic-binding capacity as well; but it is probable that such a change, if present, would merely elevate the serum BSP concentration at which the slow phase became manifest and would not produce a change in the final slope of the curve.

SUMMARY

Two 17-substituted testosterones in common clinical use (methyltestosterone and norethandrolone), administered orally in doses ranging from 20 to 100 mg. per day, have been observed to produce marked elevations in 45-minute BSP retention and corresponding reductions in BSP clearance in all subjects treated with these agents. These effects were not observed with testosterone propionate at either equimolecular or equivalent anabolic doses. The BSP changes were unaccompanied by clinical jaundice, or by alterations in any of several common laboratory indices of liver function. Maximum depression of serum BSP clearance occurred within 7 to 10 days after starting

therapy, and conversely normal clearance was usually restored within a similar period after stopping treatment. Indirect evidence indicates that the defect involves the hepatic excretory mechanism rather than liver dye uptake. The significance of this change is uncertain, but it is presumed not to represent liver damage.

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SERUM ISOCITRIC DEHYDROGENASE ACTIVITY WITH PARTICULAR REFERENCE TO LIVER DISEASE

R. L. Sterkel, M.D., Jean A. Spencer, M.D., S. K. Wolfson, Jr., M.S., and H. G. Williams-Ashman, Ph.D.*
Chicago, Ill.

RECENTLY many reports have described large changes in the activity of a number of serum enzymes in hepatic disease. Diagnostic implications concerning the type and extent of hepatocellular damage have been ascribed to the levels in serum of glutamic oxalacetic transaminase (GOT),[1-4] glutamic pyruvic transaminase (GPT),[5] lactic (LD) and malic dehydrogenases,[6-8] aldolase,[9, 10] and phosphohexoisomerase.[10, 11] Most noteworthy were dramatic elevations in the activity of most of these enzymes in the sera of patients with hepatitis of viral origin.

The recent discovery of certain triphosphopyridine nucleotide (TPN)-specific enzymes in human blood serum[12, 13] led to a survey of their activity in various disease states. In particular, serum isocitric dehydrogenase (ICD) levels were found to be increased in some hepatic diseases.[12] This paper describes changes in the activity of serum ICD in a variety of liver disorders in relationship to parallel alterations of two other serum enzymes which have been studied previously in these clinical situations, namely, LD and GPT. The diagnostic and prognostic implications of these findings are discussed.

METHODS AND MATERIALS

Blood samples were obtained from the cubital vein without regard to the fasting state. Anticoagulants were not employed. The blood was allowed to clot at room temperature, and was centrifuged in polyethylene tubes within one hour after collection. The sera were centrifuged again to ensure complete removal of erythrocytes. Sera showing the slightest visible trace of hemolysis were invariably discarded.

Serum ICD activity was determined by the method of Wolfson and Williams-Ashman.[13] GPT activity was estimated by the spectrophotometric procedure of Wroblewski and LaDue.[14] The lactic dehydrogenase added in excess to the GPT test system was purified from rabbit muscle, and was devoid of GPT activity. Serum LD levels were measured by a modification of the method of Kubowitz and Ott.[15] The total volume of the reaction mixture was 3.0 ml. and the final concentrations of the ingredients were as follows: 0.033 M sodium phosphate buffer of pH 7.4; 0.0033 M potassium pyruvate; and 0.0001 M dihydrodiphosphopyridine nucleotide (DPNH). In the majority of experiments the reactions were carried out at 25 ± 0.5° C. as described elsewhere.[13] In the few cases where the temperature at which the reactions were run was other than 25° C., the rates of

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*Scholar in Cancer Research of the American Cancer Society.

reaction were corrected to the latter temperature assuming a temperature coefficient (Q_{10}) of 1.7 over the range 20 to 30° C. In each test system, the change in absorbency at 340 mμ was determined at a number of time intervals. The initial and final absorbency at 400 mμ was also measured in order to ensure that nonspecific changes due to turbidity did not occur. The rates of reaction were calculated from the linear portions of plots of change in absorbency at 340 mμ versus time. The millimolar extinction coefficient of DPNH and of dihydrotriphosphopyridine nucleotide (TPNH) at 340 mμ was assumed to be 6.22.

RESULTS

One unit of enzyme activity was defined as that equivalent to the transformation of 1 mμM of substrate per 1 ml. of serum per hour at 25° C. In the sera of apparently healthy adults, the activity of the 3 enzymes measured was found to be independent of age, race, and sex. The normal levels for ICD were found to be 109 ± 56 (range 47 to 264)[13] and for LD, 7,400 ± 2,020 (range 4,600 to 12,100)—a value in close correspondence with that observed by others.[6] The mean activity of serum GPT was 360 ± 130 (range 212 to 685) (cf. 5).

Determinations of ICD in serum were performed upon 250 patients. Serum LD and GPT levels were also measured in most of the subjects with hepatic disease. The changes in serum enzyme activity observed in various disease states may be summarized as follows.

Viral Hepatitis.—Serum ICD was estimated on 95 occasions in 16 individuals with infectious or serum hepatitis and 9 times in 5 patients with hepatitis complicating infectious mononucleosis. Elevations to levels far above the upper limit of the normal were noted in all instances where measurements were made within the first 10 days following the onset of jaundice (Fig. 1). The average ICD activity observed in the acute phase of hepatitis was 1,480 units (range 390 to 3,480), i.e., approximately 15 times the mean of normal. It should be emphasized that in only one instance, to be discussed below, was an ICD activity greater than 610 units observed in any disease other than viral hepatitis. In patients with the usual benign course and prompt recovery of this disease, serum ICD returned to normal at about the beginning of the third week following the onset of jaundice (Fig. 2). In 3 instances in which a chronic form of the disease ensued, as proved by liver biopsy, ICD activity remained enhanced for as long as 4 months after icterus developed. This comprised such a small group that the results were not tabulated; values of 765, 1,310, and 1,320 units were obtained at this interval. In another group, 3 patients with acute necrosis of the liver died as the result of hepatic failure within 3 weeks after the onset of viral hepatitis. Although the magnitude of the initial elevation of serum ICD was relatively high in each instance, the usual reversion toward the normal range was progressing at the time of death, and there was nothing characteristic which might have indicated a fatal prognosis.

Serum LD activity was determined on 56 occasions in the above-mentioned subjects. Elevations were noted in some cases of viral hepatitis, but as can be seen from Fig. 3, the extent of alteration in serum LD was much less than that of ICD. Similar findings concerning serum LD in viral hepatitis have been described by Hsieh and Blumenthal.[6]

GPT levels were measured in 56 samples of serum from patients with infectious or serum hepatitis and on 9 occasions in hepatitis complicating infectious mononucleosis (Fig. 4). The changes in activity of this enzyme were similar to those found for ICD, but sometimes were of even greater magnitude. Within the first 10 days after the onset of jaundice, a mean value of 18,600 units (range 500 to 43,000) was observed. In no instance was a GPT level greater than 1,800 units recorded in any condition other than the acute phase of viral hepatitis. The changes observed in serum GPT in the latter disease agree well with the findings of Wroblewski and LaDue.[5] Serum GPT activity usually returned to normal at the beginning of the fourth week of jaundice (Fig. 5).

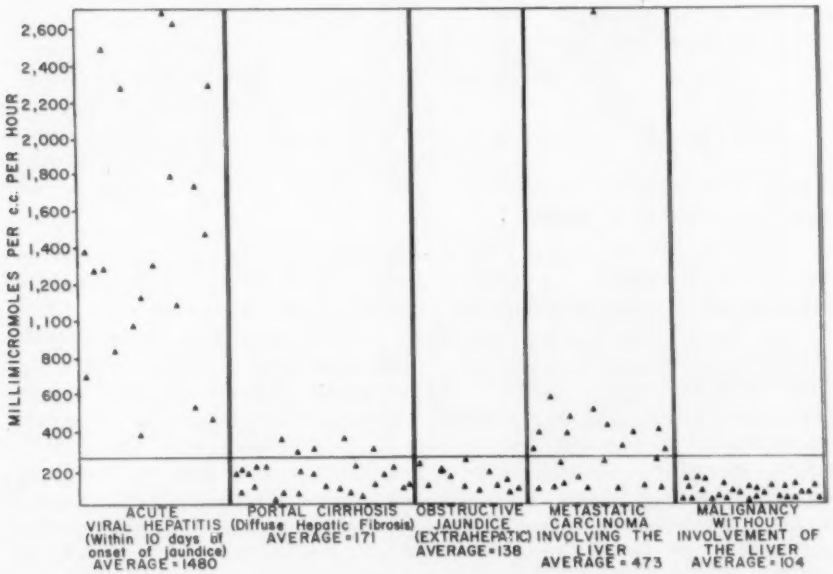

Fig. 1.—Serum isocitric dehydrogenase activity in various diseases of the liver. In Column 1 each triangle represents the serum isocitric dehydrogenase activity obtained within 10 days after the recognition of jaundice in 16 patients with viral hepatitis. Each triangle in Column 2 represents all determinations of serum ICD activity in 16 patients with portal cirrhosis. In Column 3 a triangle represents each of 12 determinations of ICD activities performed on 6 patients with obstructive jaundice. Each triangle in Column 4 is placed at the level of activity of serum ICD found on 23 instances in 21 patients in whom carcinoma had been proved metastatic to the liver. In Column 5 each triangle represents the level of serum ICD activity obtained in each of 28 cases with metastatic malignancy proved not to be involving the liver. The horizontal line at the level of 264 represents the upper level of normal activity for serum ICD. Each column includes all determinations performed in each diagnostic category.

Portal Cirrhosis.—Serum ICD activity was estimated on 27 occasions in 19 patients with portal cirrhosis. The values observed ranged from 31 to 358 units (mean 171), as shown in Fig. 1. Twenty serum GPT determinations were performed on 12 of these patients; the range was 126 to 975 units (mean 426) (Fig. 4). A similar number of serum LD determinations gave values ranging from 5,790 to 24,400 units (mean 11,700) as illustrated in Fig. 3. At the time the enzyme activities were determined, 4 of the patients were in hepatic coma and

3 expired subsequently within 2 to 19 days. The serum ICD levels were 342, 358, 100, and 114 units, respectively. Serum LD activities were 9,900, 24,000, 8,260, and 15,200 units. The serum GPT levels were 230, 500, 596, and 538

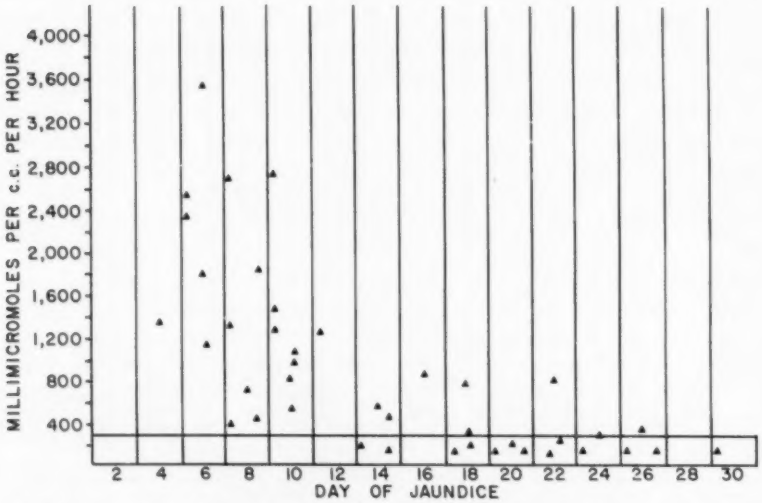

Fig. 2.—Serum isocitric dehydrogenase activity in viral hepatitis. Serial determinations of enzyme activity in 10 patients with uncomplicated viral hepatitis are illustrated, tabulating the disease from the first day of recognizable jaundice. The horizontal line at 264 represents the upper limit of normal activity for serum ICD.

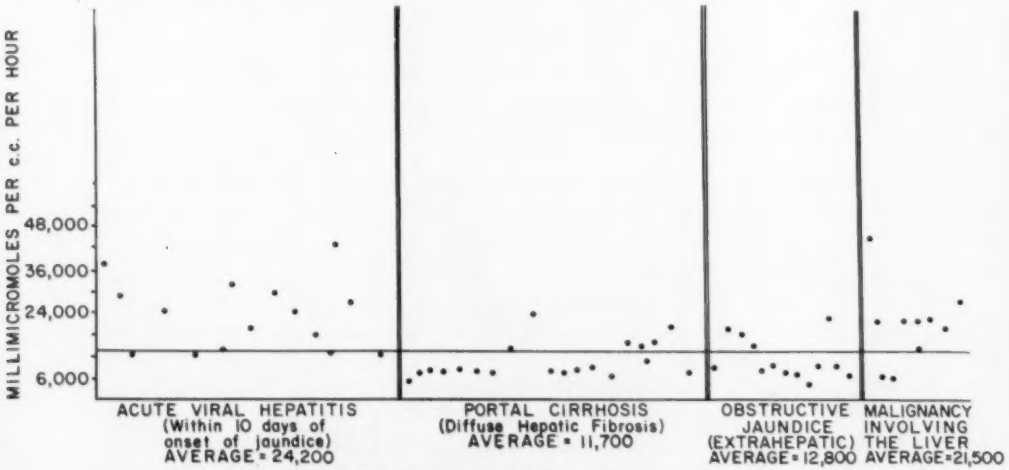

Fig. 3.—Serum lactic dehydrogenase activity in diseases of the liver. Each circle represents a value obtained for activity of serum LD. Column 1 illustrates all levels of activity determined within 10 days after the onset of recognizable jaundice in patients with viral hepatitis; Column 2, all levels of activity obtained in patients with portal cirrhosis; Column 3, all values obtained in patients with extrahepatic obstructive jaundice; Column 4, all values in patients with metastatic carcinoma proved to be involving the liver. The horizontal line at 12,100 represents the upper limit of normal activity for serum LD.

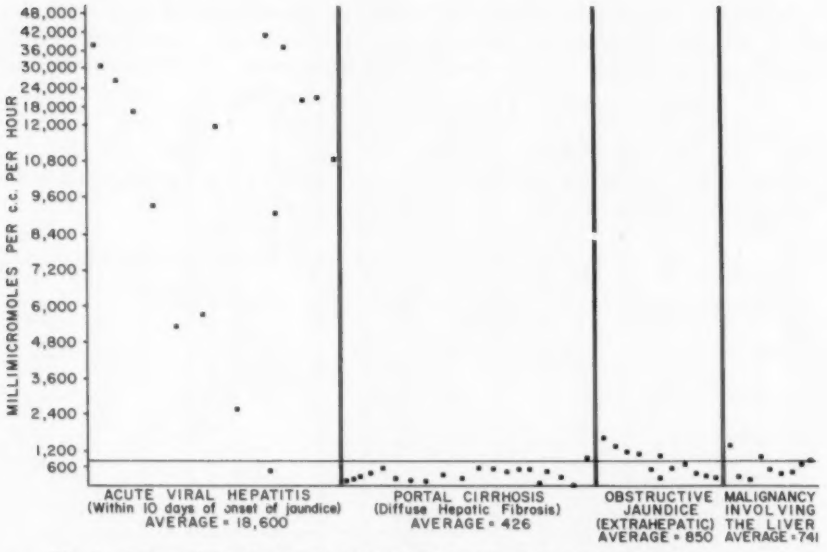

Fig. 4.—Serum glutamic pyruvic transaminase activity in diseases of the liver. As in Fig. 3, each square represents a determination of serum GPT activity. The horizontal line at 685 represents the upper limit of normal for activity of serum GPT.

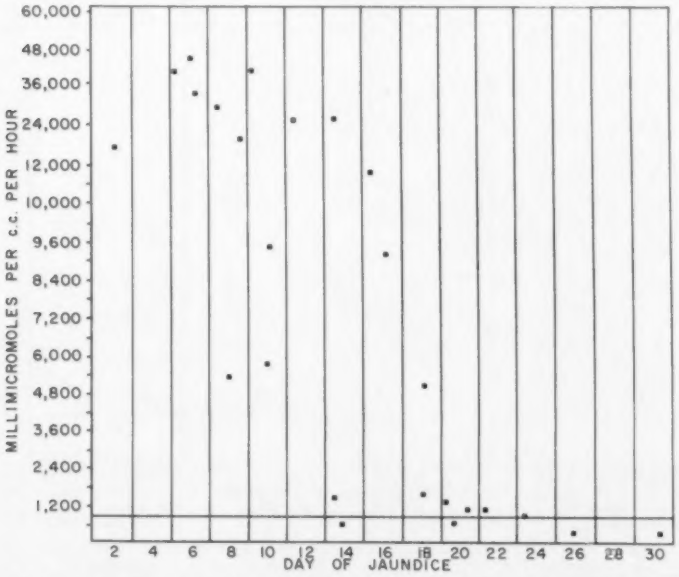

Fig. 5.—Glutamic pyruvic transaminase serum activity in viral hepatitis. Serial determinations of serum activity in 10 patients with uncomplicated viral hepatitis are illustrated, with "day of jaundice" dating from the first day of recognizable icterus. Each square represents a value for serum GPT activity. The horizontal line at 685 represents the upper limit of normal activity of serum GPT.

units. There was no relationship between the activity of serum ICD, LD, and GPT and the degree of hyperbilirubinemia or clinical evidence of the activity of the portal cirrhosis in any of these patients. Portal cirrhosis does not lead to large changes in the levels of these enzymes in serum. In the small group of patients with hepatic coma, it was impossible to assign changes characteristic of either coma or impending fatal outcome.

Obstructive Jaundice.—Serum enzyme activities were determined in 6 patients who were jaundiced as a result of extrahepatic obstruction of the common bile duct. Three of these cases resulted from a benign cause and 3 were due to malignant disease. Twelve serum ICD determinations gave values (42 to 240 units) of which all were well within the normal range. Serum LD activities ranged from 6,200 to 22,900 units (mean 12,800). The GPT activity of 13 sera varied from 289 to 1,800 units.

Malignancy With and Without Hepatic Involvement.—Serum ICD was determined in 49 patients with some form of extensive malignancy. In 21 instances liver involvement was confirmed by biopsy, either at surgery or by postmortem examination. As illustrated in Fig. 1, 23 measurements were made in this group with hepatic invasion. The values found varied from 95 to 4,500 units (mean 473). The one extreme elevation occurred in a 31-year-old woman with metastatic carcinoma of the breast. Liver metastases were demonstrated at the time of bilateral ovariectomy and adrenalectomy 75 days previously. She had received transfusions at that time and had recently experienced an onset of jaundice. The highest ICD activity in the remainder of the group was 610 uints. Approximately one-half of the patients with liver metastases exhibited relatively small elevations of serum ICD. In the group of 28 cases without liver metastases, no abnormal activities were noted. The type of malignancy studied included carcinoma of the prostate, breast, lung, stomach, colon, rectum and pancreas, malignant melanoma, lymphosarcoma, Hodgkin's disease, myelogenous and stem-cell leukemia, osteogenic sarcoma and glioblastoma multiforme. Elevations of serum ICD in an individual with a malignancy appear to indicate liver involvement.

Serum LD and GPT levels were raised occasionally in patients with metastatic disease of the liver (Figs. 3 and 4) as has been noted by others.[5, 6, 16]

Miscellaneous Diseases.—Serum ICD activities were estimated in 7 patients with electrocardiographic confirmation of large acute myocardial infarctions. The assays were performed within 24 hours after the onset of symptoms in 4 of these cases and the values obtained fell well within the normal range. Serial determinations of serum ICD in all of these patients clearly demonstrated that myocardial infarction does not result in abnormal levels of this serum enzyme.

Determinations of serum ICD were performed in cases of decompensated and arteriosclerotic heart disease, diabetes mellitus, pernicious anemia, hypogammaglobulinemia, chronic ulcerative colitis, thyrotoxicosis, viral pneumonia, breast abscess, Cushing's syndrome, chronic relapsing pancreatitis, acute pyelonephritis, acute prostatitis, diabetic nephropathy, active rheumatoid arthritis, pheochromocytoma, acute intermittent porphyria, pulmonary tuberculosis and

in patients receiving massive ACTH and corticosteroid therapy. In no instance were values outside the normal range observed. It has been recorded elsewhere[13] that serum ICD is within the normal range in the sera of term pregnancies.

DISCUSSION

The magnitude and sequence of changes in the activity of serum ICD and GPT in viral diseases of the liver is about the same. However, measurements of serum ICD have several advantages over those of transaminases. The ICD assay system measures a single step reaction, the kinetics of which can be followed spectrophotometrically in a single cuvette. In the commonly employed spectrophotometric procedure for GPT, use is made of a coupled reaction in which the pyruvate formed by the action of this serum transaminase is determined by oxidation of DPNH in the presence of excess lactic dehydrogenase.[5] Thus a decrease in absorbency at 340 $m\mu$ is measured, whereas in the ICD test system an increase in absorbency is determined. In the ICD reaction spectrophotometric readings are, therefore, made on the more sensitive range of the spectrophotometer. Moreover, the presence of pyruvate in sera makes necessary a preliminary preincubation in the GPT assay system which is not necessary in the measurement of serum ICD activity. In our experience TPNH, the product of ICD action, is stable in the presence of serum at neutral pH, unlike DPNH.

With a number of sera from patients with acute hepatitis, the relationship of the amount of serum added to the ICD test system to the rate of reaction was studied, and was found to be linear in every case. This suggests that the elevated enzyme activities observed reflected a true increase in the content of ICD in the serum rather than the presence of some activator or the removal of some inhibitory substance. The recent claim[17] that there are two distinct components in serum which possess LD activity suggests that interpretation of changes in the activity of oxidizing enzymes in unfractionated serum in various disease states may be more complex than is often assumed. However, in the absence of evidence to the contrary, it is reasonable to assume that the elevated serum ICD levels in patients with acute hepatitis results from the outflow of one discrete enzyme from hepatic cells into extracellular fluids.

The determination of serum ICD activity has two main clinical advantages over the determination of serum SPT in liver disease. First, as yet we have been unable to demonstrate elevations of serum ICD activity in any disease in which the liver parenchyma is not involved. This has occurred in spite of the fact that the ICD activity of some other organs has been demonstrated to be of order of magnitude as the transaminases. Of particular interest in this connection is our failure to demonstrate elevations in serum ICD activity in cases with acute myocardial infarction in spite of the fact that we have observed[18] in human tissues obtained within 10 hours post mortem that the ICD activity of cardiac muscle is of the same order of magnitude as that of liver. ICD activity in human serum is increased greatly after acute liver injury but not in acute myocardial infarction. Clearly factors other than tissue content can determine the magnitude of increase of the activity of a serum enzyme following specific organ damage. Further, elevations of ICD in hepatitis complicating infectious

mononucleosis, in which situation no significant hepatocellular necrosis can be demonstrated hstologically,[19] suggests that cellular damage not involving necrosis may allow increased release of cellular enzymes into the serum.

The other clinical advantage in determination of serum ICD activity over that of GPT suggested by our findings is the differentiation of intrahepatic and extrahepatic obstructive jaundice. In patients with toxic and viral hepatitis presenting the usual laboratory criteria for obstructive jaundice (elevation of serum bilirubin, alkaline phosphatase, and cholesterol levels with normal values in the serofloceulation studies), we found elevations of ICD and GPT in the serum. However, we were unable to demonstrate increased activity of ICD in the serum of any of our patients with extrahepatic obstruction, whereas the activity of GPT in the serum of some of these cases was above the normal limits (Figs. 1 and 4). Therefore, under these circumstances determination of serum ICD activity appears to yield more specific information than does that of serum GPT.

SUMMARY

The isocitric dehydrogenase activity of human blood serum is increased from four- to thirty fold in the early stages of acute hepatitis of viral origin. The serum levels of this enzyme are also elevated, although to a lesser extent, in some malignancies involving metastases to the liver, but are well within the normal range in cirrhosis of the liver and in extrahepatic obstructive jaundice. No change in the activity of serum isocitric dehydrogenase was observed in a wide variety of other diseases, including myocardial infarction. The changes in serum isocitric dehydrogenase in acute hepatitis resemble parallel alterations of serum glutamic pyruvic transaminase, but are of much greater magnitude and follow a different course from changes in serum lactic dehydrogenase. These findings, therefore, indicate that serum isocitric dehydrogenase activity may be of value in recognizing the presence of acute hepatic injury, in following the course of viral hepatitis, in detecting metastatic malignancy involving the liver, and in differentiating between intrahepatic and extrahepatic obstructive jaundice.

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SERUM ENZYMES IN DISEASE

IV. Lactic Dehydrogenase and Glutamic Oxalacetic Transaminase Levels In Renal Disease

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INTEREST in serum enzymes has been primarily related to their value as indicators of cardiac, hepatic, muscle, and neoplastic disease.[1-4]

References to the results obtained in patients with renal disease have been few. White[5] has reported elevated lactic dehydrogenase (L.D.), but normal glutamic oxalacetic transaminase (GOT) levels in 3 patients with "nephritis." Wacker, Ulmer, and Vallee[6] have stated that the L.D. level is not elevated in "simple" uremia resulting from chronic renal disease, but may be increased in patients with infarction of the kidney or with subacute glomerulonephritis. Hsieh and Blumenthal[7] found elevated L.D. levels in 3 of 6 patients with chronic nephritis. The 3 patients with the elevated L.D. levels, however, had normal levels of nonprotein nitrogen, while the 3 with normal enzyme levels had azotemia. In an earlier report from this laboratory,[3] 6 of 8 patients with various chronic renal diseases had elevated L.D. levels. Acute and chronic renal disease, azotemia, and uremia have been found[8-10] to be associated with normal levels of glutamic oxalacetic transaminase. However, in the experimental production of graded renal infarcts in dogs by arterial ligation, the GOT has been reported to rise above normal levels in proportion to the size of the infarct.[11]

In the present study, an attempt has been made to clarify the factors responsible for serum enzyme abnormality in patients with renal disease. The results demonstrate an appreciable incidence of abnormal lactic dehydrogenase levels, but rare elevations of glutamic oxalacetic transaminase levels in patients with renal disease. No single factor responsible for the frequent occurrence of elevated L.D. levels in patients with renal disease could be identified.

MATERIALS AND METHODS

Seventy-one patients with various types of renal disease were studied. All but 8 patients were males. They ranged in age from 15 to 78. All of the patients had one or more L.D. determinations for a total of 181. Of the 71 patients, 63 patients had one or more GOT determinations. A total of 139 GOT determinations were performed. Nonprotein nitrogen determinations were performed at the approximate time of the serum enzyme determinations. Also, most patients had multiple determinations of serum albumin, cholesterol, carbon dioxide combining power, and 24-hour urinary protein excretion. Autopsies or renal biopsies were

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performed in 35 of the 71 patients. Patients who were believed to be in congestive heart failure were not included in this study, since previous studies have shown that L.D. elevation may occur in heart failure.[18] There was clinical or biochemical evidence of hepatic disease in 1 patient with cirrhosis and in the 3 patients with a diagnosis of carbon tetrachloride poisoning.

In the group with "collagen disease," 10 patients had a diagnosis of systemic lupus erythematosus and 1 of periarteritis nodosa. In the miscellaneous group, there was 1 patient with hydronephrosis, 1 with papillary necrosis, 1 with polycystic kidneys, 2 with unilateral kidney agenesis, 1 with azotemia associated with cirrhosis, 2 with diabetic nephropathy and 2 with acute pyelonephritis.

In the animal experiments, mature white rabbits were employed. Both ureters were ligated as they entered the urinary bladder. A ligature was placed around the ureters at the time of surgery and was not tightened until it had been ascertained that the surgery had not produced serum enzyme elevations. Blood was obtained by cardiac puncture. The normal range of blood urea nitrogen in rabbits was found to be 10 to 20 mg. per 100 ml. The levels of L.D. and GOT before operation were in the normal human range with the exception of 1 GOT value of 46 and 2 L.D. values of 350 and 390, respectively.

The L.D. was performed by a method reported elsewhere.[3] Normal values for this enzyme are below 270 μM $DPNH_2$ per 100 ml. serum. The GOT was initially performed by the method of Karmen.[12] In the latter portion of the study, the modification of Reitman and Frankel[13] of the method of Umbreit[14, 15] was utilized. In the range of GOT values observed in the present study, no significant differences between the results obtained by the two methods could be demonstrated. Normal values for this enzyme (GOT) are below 40 units.

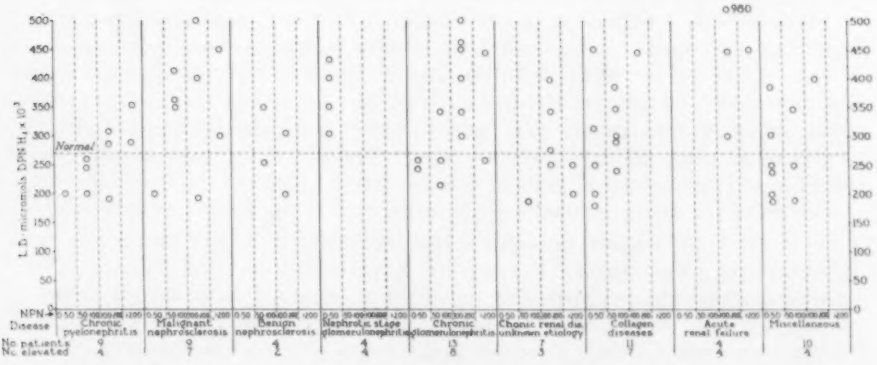

Fig. 1.—Lactic dehydrogenase levels in 71 patients with renal disease by diagnostic category and levels of NPN. The highest L.D. value obtained in each patient is represented by a circle.

RESULTS

General.—Seventy-one patients with renal disease were studied. In 43 of these, the L.D. levels were elevated at some time during their course. As may be seen from Fig. 1, elevations of the lactic dehydrogenase levels were seen in patients in all diagnostic categories.

Glutamic oxalacetic transaminase determinations were performed in 63 of the 71 patients. This serum enzyme was elevated in only 6 of the patients. The elevations were seen in one patient with advanced cirrhosis and azotemia and in 4 patients with acute renal failure. Three of the 4 with acute renal failure had a diagnosis of carbon tetrachloride poisoning and associated hepatic

necrosis. The fourth patient with acute renal failure was an alcoholic in delirium tremens whose renal disease was shown by renal biopsy to represent tubular necrosis. There was no evidence of cirrhosis. The sixth patient had a diagnosis of benign nephrosclerosis with no clear explanation for the acute azotemia nor of the slightly elevated transaminase level (60 units).

Relationship to Diagnosis and Azotemia.—Lactic dehydrogenase elevations were seen in patients with and without azotemia (Figs. 1 and 2). As may be seen from these figures, there was no clear relationship between the height of the NPN and the lactic dehydrogenase level in the various types of renal disease.

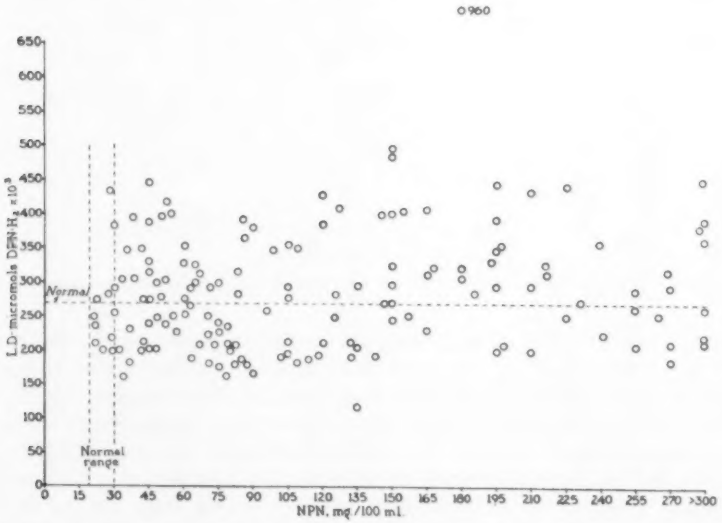

Fig. 2.—Lactic dehydrogenase levels of each patient plotted against nonprotein nitrogen values. No clear relationship is apparent.

The number of patients in each category studied, however, was small. It is of interest that all 4 patients with a diagnosis of the nephrotic stage of glomerulonephritis had elevated levels of serum L.D. None had azotemia at the time of the study. The 4 patients with acute renal failure had elevated L.D. levels. Seven out of 9 patients with malignant nephrosclerosis had elevated L.D. levels. There were 11 patients with "collagen disease"; 7 of these had elevated serum L.D. levels (Fig. 1).

Relationship to Other Clinical Findings.—There was no apparent relationship between the severity of the anemia and the occurrence of L.D. elevations. Likewise, there was no correlation between the degree of acidosis, as measured by the carbon dioxide combining power, and the level of the serum lactic dehydrogenase. The occurrence of elevated levels of this enzyme in 4 patients with the nephrotic syndrome led to the analysis of the relationship between the degree of proteinuria and the serum albumin levels and serum cholesterol levels.

There was no clear correlation between the L.D. and the degree of proteinuria and cholesterol levels, respectively (r [proteinuria] = +0.08; r [cholesterol] = +0.14). The serum albumin levels, however, showed an inverse correlation with the L.D. levels which was statistically significant ($r = -0.26$; $p < 0.05$) (Fig. 3).

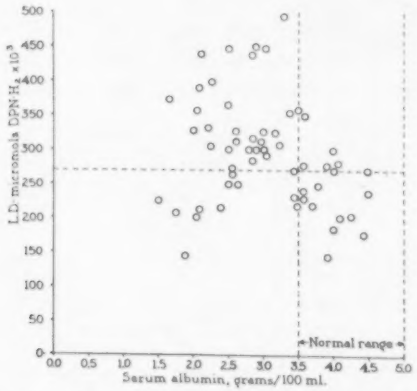

Fig. 3.—Lactic dehydrogenase levels of each patient plotted against the serum albumin levels ($r = -0.26$).

Animal Studies.—The effect of ureteral ligation on serum enzyme levels was studied in 7 rabbits (Fig. 4). Surgical trauma produced little or no elevation of the serum lactic dehydrogenase and glutamic oxalacetic transaminase levels. With bilateral ureteral ligation, there was a prompt and progressive elevation of the blood urea nitrogen level. In several of the animals, elevation of BUN was noted after ligature application and even before it was tightened. The animals survived 1 to 4 days after ureteral ligation as may be seen from Fig. 4. Elevation of the lactic dehydrogenase levels occured in only 2 of the 7 rabbits. The degree of azotemia in these, however, was no greater than that observed in some of the other animals without serum enzyme elevation. In none of the 7 animals did elevation of the GOT occur. In 3 additional rabbits, dehydration was produced by complete water deprivation for 8, 10, and 12 days, respectively (Fig. 5). A progressive rise in the L.D. was observed in only one animal. Although azotemia was noted in all, only in Rabbit 3, did the L.D. increase in parallel with the rising azotemia. It is of interest to note that this animal survived longer than the others and had a greater degree of azotemia at the time of the rise in serum L.D. levels. GOT elevations were observed only in Rabbit 2 and occurred so irregularly as to be ascribed to the trauma of the cardiac puncture performed to obtain blood.

DISCUSSION

Elevated serum lactic dehydrogenase levels were frequent in this group of patients with renal disease. This is in keeping with our previous report,[5] and with the results of others.[5-7] Elevated enzyme levels occurred in patients with

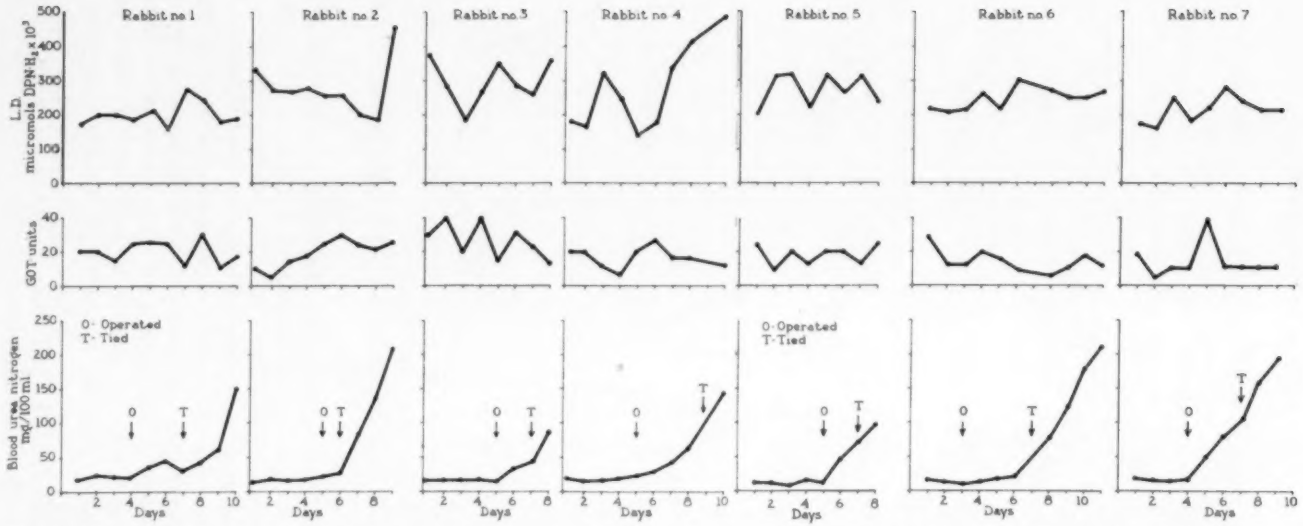

Fig. 4.—The effect of ureteral ligation on the serum enzyme levels and blood urea nitrogen values in 7 rabbits. The lactic dehydrogenase values became elevated only in Rabbits 2 and 4.

and without azotemia. Furthermore, the experimental production of renal failure in animals did not result consistently in serum enzyme elevations. Acidosis, anemia, hypercholesterolemia, and proteinuria, respectively, had no consistent relationship to the occurrence of elevated L.D. levels. There was, however, a statistically significant inverse correlation between the level of serum albumin and the serum L.D. levels. This observation is reminiscent of the demonstration by Vorhaus and Kark[16] that the serum cholinesterase level is elevated in patients with nephrotic syndrome, although depressed in other hypoalbuminemic states. These workers suggested that in patients with nephrotic syndrome the rate of albumin synthesis is increased and that this increased synthesis also is reflected in increased cholinesterase synthesis.

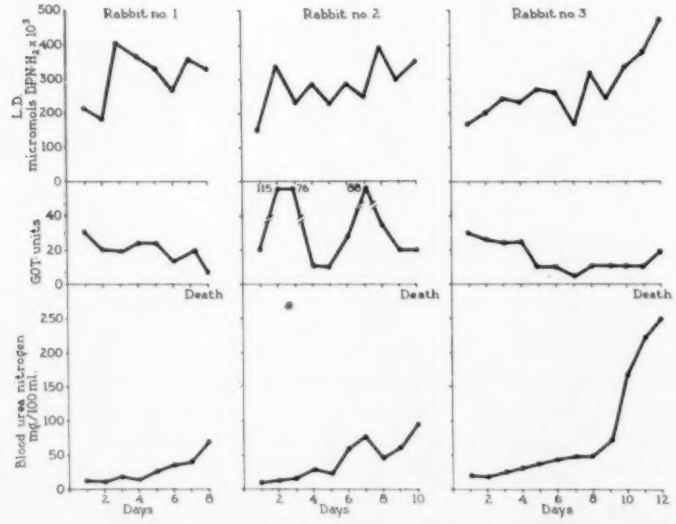

Fig. 5.—The effect of dehydration on the serum enzyme and blood urea nitrogen levels in 3 rabbits. A progressive rise in L.D. occurred only in Rabbit 3 which had the longest (12 days) and greatest degree of azotemia.

The occurrence of serum enzyme elevations in some patients with renal disease raised the possibility that the enzyme might be normally excreted in the urine and that renal disease, therefore, might interfere with its excretion. Indeed, such a relationship exists between serum amylase and renal failure.[17] In contrast, lactic dehydrogenase cannot be demonstrated in the urine of patients with normal or high serum L.D. levels.[18] Furthermore, as has been stated above, there was no apparent correlation between the degree of azotemia and the serum lactic dehydrogenase level. In animals, ureteral ligation did not uniformly result in elevations of the levels of enzyme. It should be emphasized that the animal experiments were acute. It would appear that elevated lactic dehydrogenase levels in patients with renal disease cannot be ascribed to failure of a normal route of excretion of this enzyme.

Similarly, Sibley, Fleisher, and Higgins[19] were unable to elucidate the significance of elevated serum aldolase in rats after bilateral nephrectomy and after bilateral ureteral ligation.

The serum glutamic oxalacetic transaminase levels were elevated in only 6 of the patients in the entire group. In 4 of these patients, there was associated hepatic disease. The fifth had associated delirium tremens, a condition in which the levels of several serum enzymes have been noted to be elevated.[4, 18] In only one patient with azotemia, but without apparent hepatic disease, were levels of this enzyme elevated.

Renal tissue has been shown to contain large amounts of L.D.[20] and GOT.[21] Experimentally induced necrosis of kidney tissue has been shown to produce elevation of serum GOT levels,[11] but we are not aware of similar studies with respect to serum L.D. levels. If the elevation of serum L.D. levels in the patients with renal disease were the result of release of the enzyme from necrotic or altered kidney tissue, GOT elevations might be expected in the same group of patients. This was not the case. Furthermore, there was no consistent correlation between the primary renal diagnosis and the occurrence of L.D. elevation.

The significance of the occurrence of elevated levels of lactic dehydrogenase in patients with renal disease is not clear, but this interesting phenomenon warrants further study. For clinical purposes, it may be useful to note that in patients with renal failure, elevated serum levels of lactic dehydrogenase, aldolase, and amylase may occur.

SUMMARY AND CONCLUSION

1. Forty-three of 71 patients with renal disease were found to have elevated lactic dehydrogenase levels in the serum. Although azotemia was common in this group, there was no direct relationship between the serum enzyme levels and the degree of azotemia. The degree of anemia, acidosis, proteinuria, and hypercholesterolemia could not be related to the serum lactic dehydrogenase levels. There was a statistically significant ($p < 0.05$) inverse correlation between the serum albumin levels and the lactic dehydrogenase levels.

2. Experimental renal failure produced in rabbits by ureteral ligation or by dehydration resulted in an inconstant occurrence of elevated serum lactic dehydrogenase.

3. The serum glutamic oxalacetic transaminase levels were found to be elevated in only 6 patients of 63 with renal disease. Four of these 6 had coincidental hepatic disease adequate to account for the elevated glutamic oxalacetic transaminase levels. Another had delirium tremens and acute renal failure. In one patient with renal failure, but no demonstrable hepatic disease, this enzyme was also found to be elevated.

4. The factor(s) responsible for the elevated serum lactic dehydrogenase levels in patients with renal disease could not be elucidated.

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EFFECT OF POSTURE ON SALT AND WATER RETENTION. I

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MANY physiologic changes occur when man assumes the erect position. Among these is a decrease in renal excretion of water, sodium, potassium, and chloride. Several mechanisms have been suggested to explain these effects.

The present experiments were designed to utilize a simple and reliable method for evoking the reduction in water, sodium, potassium, and chloride excretion by the kidneys on quiet standing and to elicit information as to why these changes occur. These experiments were also designed to determine whether the antidiuretic response is due to stimulation of the neurohypophysis to release an antidiuretic substance and also to attempt to see what part was played in postural salt retention by the adrenal cortex, the lower extremities, and congestion of the head.

METHODS

The subjects for this experiment were all normal men from 20 to 60 years old with the exception of the particular patients, noted below, who were used for certain specific experiments. Both normal subjects and patients were on their regular diets prior to the experiments and were in the fasting state during the experiments. They were given 200 to 400 ml. of water on arising to compensate for possible water deficit which may have occurred during sleeping. This has been found by our experience to be advisable in order to bring out the sharp contrast between the reclining and upright positions. Smoking was prohibited. The experiments were done soon after the subjects awakened in the morning. All voided in the upright position.

Each experiment was divided into five 40-minute periods. Each subject drank 1.3 ml. of 0.2 per cent sodium chloride per pound of body weight, as previously recommended by Epstein and associates[1] before each period and the urine was collected at the end of each. They were recumbent during the first 2 periods (several who did not have a good water diuresis remained lying for 3 periods), stood quietly for the third, and were recumbent for the last 2. The volume and concentration of sodium, potassium, and chloride were measured on each urine specimen. The sodium and potassium concentrations were determined with a Baird standard internal flame photometer and chloride was determined by the method described by Schales.[2]

The subjects consisted of several groups in which various factors, relating to the postural retention of water and salt, were investigated. These groups were made up of the following: (1) 7 normal men, aged 28 to 57, were control subjects; (2) 6 control subjects had 90 ml. of 100 proof Bourbon whiskey substituted for 90 ml. of 0.2 per cent NaCl 5

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minutes before they assumed the upright position; (3) 4 patients with Addison's disease who were being maintained on cortisone; (4) 2 patients who had sustained bilateral high thigh amputations months previously: one following trauma, the other following a saddle embolus presumably from a left ventricular mural thrombus after a coronary occlusion with myocardial infarction; (5) 3 normal medical students, around whose necks a blood pressure cuff was inflated to 20 mm. Hg during the standing period; (6) one patient with the superior mediastinal syndrome, before and after therapy.

An 11-year-old boy with epispadias, otherwise normal, with an indwelling catheter in place was given 0.2 per cent NaCl while recumbent until diuresis occurred. He then stood quietly and urine samples were collected every 5 minutes to determine the interval before the onset of salt and water retention. He then lay down and urine samples were collected every 5 minutes to determine the duration of the effects of standing. During this experiment the subject drank 200 ml. of 0.2 per cent NaCl every 40 minutes.

RESULTS

1. *Postural Antidiuresis and Antisaluresis.*—The data collected on the young boy with epispadia are shown in Table I. The decrease in water, sodium, and potassium excretion is evident between 5 and 10 minutes after assuming the erect position. This effect persists for 25 minutes after resuming the recumbent position.

TABLE I. SHOWING TIME OF ONSET AND PERSISTENCE OF POSTURAL ANTIDIURESIS AND ANTISALURESIS IN AN 11-YEAR-OLD BOY WITH INDWELLING CATHETER IN A DIURESIS WITH 0.2 PER CENT NaCl

TIME (MIN.)	POSITION	URINE FLOW (ML./MIN.)	Na EXCRETION (mEq./MIN.)	K EXCRETION (mEq./MIN.)
0	Lying	1.8	0.094	0.063
5	Lying	3.0	0.186	0.110
10	Lying	3.8	0.213	0.114
15	Lying	5.3	0.198	0.091
20	Lying	6.1	0.158	0.070
5	Standing	5.0	0.120	0.052
10	Standing	1.6	0.040	0.017
15	Standing	0.5	0.014	0.006
20	Standing	0.3	0.009	0.004
25	Standing	0.6	0.017	0.008
30	Standing	0.4	0.025	0.007
35	Standing	0.4	0.013	0.007
40	Standing	0.5	0.017	0.010
5	Lying	0.5	0.018	0.011
10	Lying	0.6	0.023	0.022
15	Lying	0.5	0.025	0.019
20	Lying	1.0	0.051	0.040
25	Lying	1.4	0.093	0.060
30	Lying	3.0	0.255	0.129
35	Lying	6.2	0.334	0.127
40	Lying	7.9	0.164	0.055

2. *Control Subjects.*—The data obtained in the control subjects are shown in Table II. These results show the decrease in water, sodium, and chloride excretion on quiet standing. These is an equally marked decrease in potassium excretion. These data show also that the decrease in excretion of water and electrolytes is sometimes more evident in the first recumbent position after standing than in the standing period. This may be due to a delay in the onset of the decreased excretion of water and electrolytes on standing and persistence

of the effects of standing for 25 minutes after assuming the recumbent position (Table I). With the marked change in urine flow, this delay is emphasized by the dead space of the urinary collecting system. In one subject (B. M. D.) even though there was no diuresis before standing, standing caused a decrease in urine flow of 43 per cent. In this subject the response to standing of sodium, potassium, and chloride excretion is similar to that observed in other controls.

TABLE II. NORMAL CONTROL SUBJECTS

SUBJECT	POSITION	TIME (MIN.)	URINE VOLUME (ML.)	URINE (ML./ MIN.)	Na (mEq./ MIN.)	Cl (mEq./ MIN.)	K (mEq./ MIN.)
J. E. W.	Lying	40	335	8.39	0.226	0.169	0.051
	Standing	40	72	1.80	0.074	0.059	0.018
	Lying	40	142	3.53	0.199	0.152	0.035
J. L. C.	Lying	40	44	1.10	0.055	0.090	0.079
	Lying	40	360	9.00	0.081	0.156	0.119
	Standing	40	270	6.76	0.013	0.058	0.074
	Lying	40	50	1.25	0.030	0.055	0.047
	Lying	40	450	11.25	0.113	0.146	0.113
W. P. W.	Lying	40	140	3.50	0.053	0.136	0.091
	Lying	40	480	12.00	0.048	0.156	0.120
	Standing	40	145	3.62	0.018	0.078	0.071
	Lying	40	105	2.63	0.037	0.159	0.072
	Lying	40	365	9.13	0.091	0.157	0.114
B. M. D.	Lying	41	26	0.64	0.107	0.180	0.063
	Lying	41	34	0.83	0.157	0.212	0.070
	Standing	40	19	0.47	0.078	0.135	0.055
	Lying	40	29.5	0.74	0.099	0.151	0.068
	Lying	40	183	4.60	0.197	0.270	0.101
C. H. W.	Lying	40	97	2.40	0.229	0.275	0.136
	Lying	40	530	13.20	0.172	0.292	0.166
	Standing	40	360	9.00	0.099	0.198	0.135
	Lying	40	72	1.80	0.108	0.157	0.102
	Lying	40	360	9.00	0.234	0.307	0.153
E. E. G.	Lying	40	217	5.40	0.119	0.146	0.057
	Lying	40	465	11.60	0.110	0.314	0.105
	Standing	40	57	1.42	0.061	0.142	0.081
	Lying	40	91	2.27	0.093	0.187	0.103
	Lying		380	9.50	0.123	0.341	0.133
H. H. H.	Lying	40	40	1.00	0.144	0.200	0.057
	Lying	60	280	4.67	0.070	0.144	0.061
	Standing	40	205	5.13	0.082	0.155	0.067
	Lying	40	95	2.38	0.073	0.092	0.073
	Lying	40	390	9.75	0.093	0.212	0.135

Another subject (H. H. H.) is included in the series to show a variation in response which occurred when a subject was allowed to do the experiment with personal responsibility for timing and collecting the specimens. This subject is a tense, conscientious person in whom, presumably, emotional factors altered the expected response. We have found that unless this simple procedure is supervised carefully and the subject is without personal responsibility, the usual response cannot be obtained with certainty.

3. *Effect of Alcohol Ingested Just Before Standing.*—The data on 6 of the control subjects who ingested 90 ml. of 100 proof Bourbon whiskey 10 minutes

prior to standing are shown in Table III. These data illustrate that alcohol inhibits the antidiuresis of quiet standing but has no definite effect on the excretion of sodium and chloride. This is not the case with potassum excretion. In the alcohol experiments, potassium excretion decreased on standing, as did sodium and chloride excretion; however, on resuming the reclining position the excretion of potassium continued to be decreased, whereas the excretion of sodium and chloride returned to approximately prestanding levels. In the control experiments potassium excretion was similar to sodium and chloride excretion through all phases of the experiment. We have no explanation for this observation.

TABLE III. EFFECT OF ALCOHOL INGESTION JUST BEFORE STANDING

SUBJECT	POSITION	TIME (MIN.)	URINE VOLUME (ML.)	URINE (ML./ MIN.)	Na (mEq./ MIN.)	Cl (mEq./ MIN.)	K (mEq./ MIN.)
J. L. C.	Lying	40	81	2.03	0.131	0.219	0.121
	Lying	40	478	11.94	0.107	0.259	0.185
	Standing	40	398	9.94	0.040	0.129	0.114
	Lying	50	438	8.75	0.061	0.114	0.039
	Lying	30	122	4.06	0.162	0.194	0.057
W. P. W.	Lying	40	46	1.15	0.063	0.120	0.069
	Lying	40	423	10.60	0.074	0.182	0.132
	Standing	40	385	9.6	0.029	0.083	0.063
	Lying	40	385	9.6	0.029	0.083	0.033
	Lying	40	230	5.8	0.069	0.124	0.037
B. M. D.	Lying	40	27	0.675	0.138	0.159	0.058
	Lying	50	45	0.90	0.187	0.208	0.070
	Standing	40	117	2.92	0.102	0.117	0.053
	Lying	40	349	8.73	0.087	0.113	0.061
	Lying	40	142	3.53	0.128	0.142	0.037
C. H. W.	Lying	40	27	0.70	0.066	0.106	0.065
	Lying	50	445	8.9	0.080	0.204	0.115
	Standing	40	285	7.1	0.025	0.100	0.071
	Lying	40	365	9.2	0.050	0.082	0.055
	Lying	40	355	8.9	0.115	0.169	0.057
E. E. G.	Lying	40	84	2.1	0.116	0.124	0.055
	Lying	40	405	10.1	0.142	0.365	0.122
	Standing	40	315	7.9	0.024	0.213	0.075
	Lying	40	375	9.4	0.028	0.216	0.052
	Lying	40	83	2.1	0.113	0.187	0.042
H. H. H.	Lying	40	35	0.88	0.073	0.125	0.055
	Lying	40	205	5.13	0.051	0.156	0.075
	Standing	40	400	10.00	0.046	0.130	0.070
	Lying	40	385	9.63	0.042	0.125	0.043
	Lying	40	335	8.38	0.084	0.108	0.050

4. *Effect of Quiet Standing on Subjects With Cerebral Congestion.*—The data in Table IV reveal that in 3 normal medical students a blood pressure cuff, inflated to 20 mm. Hg, around the neck during the standing period had little appreciable effect on the decrease in water, sodium, chloride, and potassium excretion of quiet standing. None of the subjects complained of discomfort during the cuff inflation.

TABLE IV. BLOOD PRESSURE CUFF INFLATED AROUND NECK (20 MM./HG) DURING STANDING PERIOD

SUBJECT	POSITION	TIME (MIN.)	URINE VOLUME (ML.)	URINE (ML./MIN.)	Na (mEq./MIN.)	Cl (mEq./MIN.)	K (mEq./MIN.)
B. M. D.	Lying	40	111	2.78	0.119	0.128	0.049
	Lying	40	250	6.25	0.144	0.121	0.035
	Standing	40	165	4.13	0.085	0.076	0.027
	Lying	40	59	1.47	0.061	0.060	0.020
	Lying	40	320	8.00	0.088	0.067	0.020
V. E. C.	Lying	40	65.5	1.64	0.150	0.179	0.101
	Lying	40	260	6.5	0.143	0.170	0.101
	Standing	40	160	4.0	0.142	0.165	0.096
	Lying	40	58	1.45	0.086	0.128	0.083
	Lying	40	245	6.14	0.133	0.168	0.130
C. R. C.	Lying	40	132	3.3	0.218	0.227	0.120
	Lying	40	280	7.0	0.271	0.261	0.138
	Standing	40	97	2.42	0.113	0.125	0.070
	Lying	40	12.5	0.31	0.040	0.040	0.020
	Lying	40	285	7.12	0.179	0.172	0.105

5. *Effect of Quiet Standing in a Patient With Superior Mediastinal Syndrome.*—The data in Table V were collected on a man who had superior mediastinal syndrome. Prior to therapy this patient had massive edema of his face and arms, a venous pressure (by the usual standard technique with the patient recumbent) of 550 mm. H_2O in both arms and 150 mm. H_2O in both legs. After nitrogen mustard therapy, the patient had a good clinical result with loss of edema of his face and arms and a venous pressure drop to 330

TABLE V. PATIENT WITH SUPERIOR MEDIASTINAL SYNDROME (BEFORE AND AFTER THERAPY)

SUBJECT	POSITION	TIME (MIN.)	URINE VOLUME (ML.)	URINE (ML./MIN.)	Na (mEq./MIN.)	K (mEq./MIN.)
Prior to therapy	Lying	30	195	6.5	0.272	0.104
	Lying	30	240	8.0	0.160	0.064
	Standing	30	224	7.5	0.134	0.063
	Lying	30	265	8.8	0.141	0.070
	Lying	30	254	8.5	0.160	0.068
Prior to therapy	Lying	40	120	3.0	0.135	0.045
	Lying	40	223	5.6	0.139	0.036
	Standing	40	300	7.5	0.105	0.045
	Lying	45	360	8.0	0.120	0.036
	Lying	40	337	8.4	0.164	0.038
After therapy	Lying	62	173	2.8	0.098	0.047
	Lying	40	373	9.3	0.112	0.042
	Standing	41	170	4.2	0.062	0.042
	Lying	41	60	1.5	0.086	0.032
	Lying	40	255	6.4	0.115	0.042

mm. H_2O in both arms. The data show that on two different days before therapy this patient had no significant decrease in water and potassium excretion and only a slight decrease in sodium excretion after quiet standing. One month after nitrogen mustard therapy this experiment was repeated and the subject had the usual decrease in water excretion and some decrease in sodium and potassium excretion on quiet standing.

6. *Effect of Quiet Standing on Subjects With Bilateral High Thigh Amputations.*—The data in Table VI reveal that these two subjects with bilateral thigh amputation responded to quiet standing or a 60 to 80 degree upright position with decrease in renal water and electrolyte excretion similar to normal subjects.

TABLE VI. PATIENTS WITH BILATERAL HIGH THIGH AMPUTATIONS

SUBJECT	POSITION	TIME (MIN.)	URINE VOLUME (ML.)	URINE (ML./ MIN.)	Na (mEq./ MIN.)	Cl (mEq./ MIN.)	K (mEq./ MIN.)
J. H.	Lying	40	44	1.1	0.220	0.305	0.091
	Lying	40	135	3.4	0.165	0.220	0.078
	Lying	40	93	2.3	0.082	0.121	0.045
	Standing	40	38	0.95	0.071	0.110	0.038
	Lying	40	100	2.5	0.073	0.119	0.043
T. A. D.	Lying	40	97	2.4	0.387	0.419	0.033
	Lying	40	Specimen inadvertently discarded				
	Lying	40	335	8.4	0.502	0.509	0.159
	Standing	40	165	4.1	0.124	0.143	0.039
	Lying	40	89	2.2	0.198	0.203	0.033

TABLE VII. PATIENTS WITH ADDISON'S DISEASE ON MAINTENANCE DOSES OF CORTISONE

SUBJECT	POSITION	TIME (MIN.)	URINE VOLUME (ML.)	URINE (ML./ MIN.)	Na (mEq./ MIN.)	Cl (mEq./ MIN.)	K (mEq./ MIN.)
W. P. S.	Lying	40	86	2.15	0.438	0.486	0.925
	Lying	40	46	1.15	0.225	0.239	0.460
	Lying	40	53	1.33	0.220	0.174	0.497
	Standing	40	33	0.83	0.115	0.144	0.390
	Lying	40	40	1.00	0.158	0.196	0.405
A. M.	Lying	40	31	0.78	0.133	0.102	0.013
	Lying	40	70	1.75	0.147	0.152	0.016
	Standing	28	53	1.89	0.057	0.066	0.014
	Lying	40	78	1.95	0.045	0.085	0.019
S. R. S.	Lying	80	103	1.3	0.133	0.147	0.086
	Standing	40	72	1.8	0.045	0.076	0.047
	Lying	40	28	0.7	0.044	0.059	0.037
	Lying	40	104	2.6	0.089	0.099	0.044
S. S. J.	Lying	42	29	0.7	0.087	0.129	0.048
	Lying	40	62	1.6	0.136	0.177	0.062
	Standing	40	35	0.9	0.074	0.109	0.043
	Lying	40	30	0.8	0.073	0.075	0.037
	Lying	40	225	5.6	0.112	0.107	0.062

7. *Effect of Quiet Standing on 4 Patients With Treated Addison's Disease.*—In view of the great influence which the adrenal cortex exerts on sodium excretion, it seemed of interest to see if postural antinaturesis would be altered in a patient with Addison's disease. The adrenal abnormalities in these subjects, even though maintained on adequate substitution therapy, excluded active adrenocortical participation in their response to quiet standing. The data obtained on these patients are shown in Table VII. Since none of these patients had received cortisone for at least 12 hours prior to the experiment, it was to be expected that they would not go into water diuresis.[3] In 2 of these

patients (S. R. S. and S. S. J.) the response, excepting the lack of water diuresis, was very similar to that observed in the control subjects. Even though in one of these subjects (W. P. S.) there was a marked drop in sodium, potassium, and chloride excretion prior to the standing period, there was a further decrease in sodium, potassium, and chloride on standing with a return to higher levels on reclining. In another of these subjects (A. M.) the response of sodium and chloride excretion on standing was similar to the normal; however, there was no change in potassium excretion on standing. This may have been due to the fact that this patient was excreting very small amounts of potassium prior to the standing period.

DISCUSSION

The method used is a simple and reliable one for evoking the decrease in water, sodium, potassium, and chloride excretion observed during quiet standing. This response to standing becomes apparent 5 or 10 minutes after the subject stands and persists 25 minutes after he lies down. That these changes persist for an appreciable time after the return to the reclining position would argue that the standing position initiates the release of a substance (possibly circulating) which continues to exert an effect for 30 minutes. One would not expect local postural renal circulatory changes to persist for this length of time. Decreases in glomerular filtration alone are insufficient to explain always the changes in sodium excretion as is pointed out by Epstein and associates.[1] A rise in filtration fraction is generally accompanied by a decrease in excretion of sodium, potassium, and chloride. This observation and the appearance of a vasoconstrictor substance in the blood on assuming the upright position are discussed in the following paper by one of us.

In our normal subjects the decrease in water excretion on standing was blocked by the ingestion of alcohol prior to standing. The decrease in sodium, potassium, and chloride excretion on standing was not appreciably affected by the alcohol. The qualitative decrease was unaffected even though quantitatively there were fluctuations. These results indicate that the decrease in water excretion is mediated differently than the decrease in electrolyte excretion. This conclusion is in agreement with that of Kleeman and associates[4] and Pearce and Newman.[5] From the work done by Epstein on the inhibiting action of ethyl alcohol on the neurohypophysis and our results it would seem reasonable to think that the antidiuresis of quiet standing is caused by the release of antidiuretic hormone by the neurohypophysis. The initiating cause of this release is not known. Verney[6, 7] demonstrated in dogs that the posterior pituitary may be stimulated to release antidiuretic hormone by an increase in osmolarity in the carotid artery. Change in electrolyte concentrations in the internal carotid is assumed to be an initiator of antidiuresis in man,[8] but is not proved to be a factor in the antidiuresis of quiet standing. The electrolyte retention of quiet standing is even less well understood. Lewis and co-workers[9] were able to increase the excretion of sodium by compression of the neck of the normal sitting subject and suggest that orthostatic antinaturesis is mediated

through volume receptors in the cephalad portion of the body. This has not been substantiated by others.[10] In our acute experiment we were unable to demonstrate an effect on the antidiuresis and antinaturesis of quiet standing by neck compression. However, the return to a more normal excretion pattern after therapy exhibited by one subject with the superior mediastinal syndrome tends to substantiate Harrison's[9] concept that the antidiuretic and antinaturetic response, to standing at least, are mediated through the cephalad portion of the body. Admittedly, the experiment on the patient with the superior mediastinal syndrome was not fully controlled but the results are highly suggestive. It is not possible to use patients with congestive heart failure with generalized high venous pressures as controls owing to the fact that most of these patients are either retaining salt and water with increasing failure or undergoing diuresis with improvement.

Two bilateral thigh amputees responded to "standing" in the same manner as did the normal individuals. Shifts in interstitial fluid in the legs do not seem to be the initiating factor in the decrease in renal excretion of water and electrolytes on standing. This does not agree with Pearce and Newman's[5] leg-wrapping experiments. In our experience, the antidiuresis of quiet standing was not inhibited by leg wrapping. These experiments are not included because of the discomfort our subjects experienced.

Welt[11] has stated that the adrenal cortex may play some role in postural salt retention. Holland and Stead[12] have concluded that it appears unlikely that stimulation of the adrenal cortex by ACTH or the production of cortisone or desoxycorticosterone glucoside is important in the striking salt retention produced by motionless standing. The decrease in potassium as well as sodium excretion on standing would tend to exclude the adrenal corticoids as being the initiators in this result of standing. If quiet standing caused increased release of adrenal cortical hormones, one would expect sodium to be retained and potassium excreted. The decrease in electrolyte excretion on standing in our treated patients with Addison's disease supports the hypothesis that the adrenal corticoids probably do not cause the antisaluresis of quiet standing. That cortisone was withheld from these patients 12 hours prior to the experiment may have influenced their response. Our data do not permit us to say whether or not the adrenal steroids are permissive for the initiation of sodium retention.

SUMMARY

Quiet standing represents a simple and dependable method for evoking decreases in renal excretion of water and electrolytes. Evidence that this decrease in water and electrolyte excretion is mediated through different pathways is presented. The hypothesis that the release of antidiuretic hormone by the neurohypophysis as a probable mechanism in the antidiuretic response is supported. We conclude from our data that the adrenal cortex and the lower extremities play little part in the acute decrease in sodium, potassium, and chloride secretion on quiet standing. We were unable to affect the excretion of sodium by congestion of the head in acute experiments.

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EFFECT OF POSTURE ON SALT AND WATER RETENTION

II. Appearance of a Circulating Vasoconstrictor Substance in the Blood on Assuming the Erect Position

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THE changes in urinary flow and composition which occur when a normal subject assumes the erect position from the horizontal have been the subject of much investigation. (A simple method by which these changes in water and salt excretion can be displayed has been reported.[1]) There is good evidence that the decrease in water excretion on quiet standing is mediated through the neurohypophysis by release of antidiuretic hormone.[1] The cause of the decrease in renal sodium and chloride excretion on quiet standing is not known. Evidence has been presented that the adrenal cortex and pooling of blood in the lower extremities have little influence in the acute experiment.[1] The decrease of water excretion and the decrease of sodium, potassium, and chloride excretion by the kidney on quiet standing appear to have a separate mechanism.[1]

Nickel and associates[2] found that an infusion of *l*-norepinephrine (8 gamma per min.) results in a decrease in sodium and potassium excretion. The decrease in sodium and potassium excretion is of the same magnitude that has been found when a subject stands quietly after being horizontal. Armin and Grant[3] have evolved a method by which they are able to detect a vasoconstrictor substance in human blood in very low concentration with the same vasoconstrictor activity as epinephrine 0.01 μg per liter.

The following study was undertaken to determine if there is a change in the vasoconstrictor activity of the blood at the same time that there is a decrease in sodium, potassium, and chloride excretion when a normal subject assumes the erect position from the horizontal.

METHOD

Three normal men, 19, 35, and 48 years of age, respectively, were used. The protocol for all 3 was essentially the same. The subject ate his usual diet on the days preceding the experiment. No smoking was allowed on the day of the experiment. The subject arrived at the laboratory after fasting overnight except for a glass of water on arising. On entering the laboratory he lay down on a comfortable couch and was kept quiet and warm and a polyethylene catheter was inserted into a suitable antecubital vein and a very slow (1 to 2 ml. per hour) infusion of normal saline was started through the catheter to assure its patency. The subject stood a moment and then passed all the urine he could;

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the time was noted, and the specimen was discarded. Each time that a urine specimen was obtained the subject stood beside the couch momentarily and, as nearly as possible, emptied his bladder. He then drank 1.3 ml. of 0.2 per cent NaCl solution per pound of body weight. At 40 minutes, the first urine specimen was collected and the subject again drank the same amount of saline solution. At 80 minutes a second urine specimen was collected and the first blood sample was withdrawn, the subject then drank the same amount of saline solution, *stood up*, and remained quietly standing for the next 40 minutes. At 120 minutes (after 40 minutes of quiet standing) the third urine sample was collected, the second blood sample was withdrawn, the same amount of saline solution was ingested, and the subject *lay down*. At 160 minutes the fourth urine sample was collected, the same amount of saline solution was ingested, and the subject remained lying down. Two hundred minutes after the start of the experiment the fifth urine sample was collected and the polyethylene catheter removed from the antecubital vein. The amount of normal saline infused by the venous catheter was noted. At no time during the experiment did the subject experience any discomfort from the venous catheter. The time schedule varied slightly, but these variations were noted and, as the urine specimens' results are reported in milliliters per minute or milliequivalents per minute, it was felt that this was a permissible variation. Fig. 1 shows graphically the experiment as it was carried out.

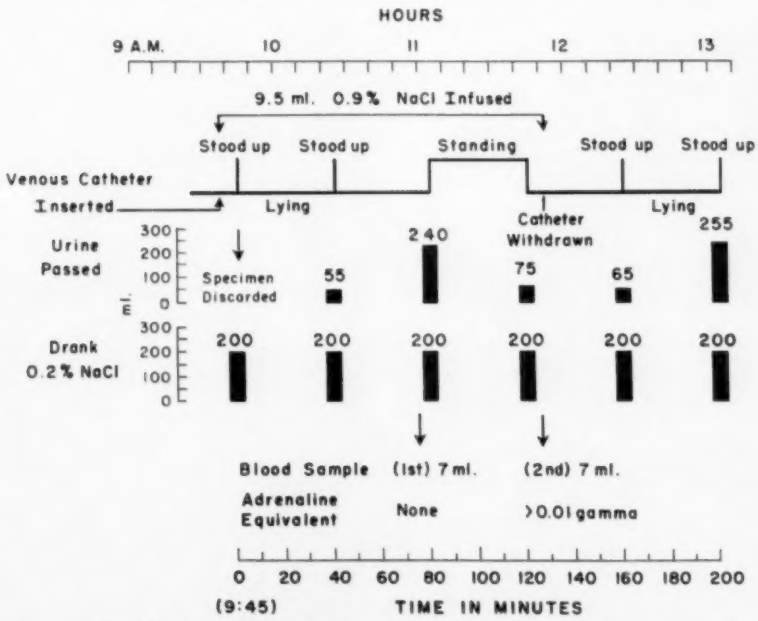

Fig. 1.—Graphic representation of a typical experiment showing the simultaneous appearance on standing of antidiuresis (antisaluresis) and increased vasoconstrictor activity in the blood.

The blood samples were withdrawn (plasma separated and ultrafiltrated) and assayed for vasoconstrictor activity as described by Armin and Grant.[3] The urine samples were measured for volume and analyzed for content of sodium, potassium, and chloride. The amount of sodium and potassium was determined by the standard internal flame photometer method; the chlorides were determined by the Conway[4] method.*

*I am indebted to J. Armin and R. T. Grant for the plasma assay and their help with these experiments. I am also indebted to Dr. Brian Macardle of the Medical Research Council for the chemical determinations.

RESULTS

The urinary volume and sodium, potassium, and chloride excretion for each of the 3 subjects' specimens are shown in Table I. These results reveal that each of the subjects had a marked decrease in his urine flow after standing. There was also a definite decrease in the sodium, potassium, and chloride excretion.

TABLE I. EFFECT OF QUIET STANDING ON THE EXCRETION OF WATER AND ELECTROLYTES

SUBJECT	POSITION	TIME	TOTAL VOLUME	VOLUME (ML./MIN.)	Na (mEq./MIN.)	Cl (mEq./MIN.)	K (mEq./MIN.)
H. E. H.	Lying	40	55	1.39	0.11	0.121	0.173
normal	Lying	42	240	5.71	0.08	0.088	0.142
man	Standing	38	75	1.98	0.05	0.042	0.074
age 48	Lying	40	65	1.63	0.05	0.040	0.070
	Lying	40	255	6.39	0.10	0.059	0.097
J. L. C.	Lying	40	195	4.87	0.10	0.112	0.074
normal	Lying	55	405	7.35	0.09	0.111	0.138
man	Standing	40	57	1.43	0.02	0.050	0.099
age 35	Lying	40	28	0.70	0.02	0.011	0.070
	Lying	40	295	7.37	0.086	0.290	0.127
D. A. N.	Lying	40	135	3.38	0.16	0.185	0.139
normal	Lying	46	370	8.05	0.16	0.181	0.147
man	Standing	35	167	4.77	0.10	0.102	0.094
age 19	Lying	41	48	1.17	0.08	0.071	0.051
	Lying	39	320	8.20	0.15	0.101	0.098

TABLE II. PLASMA ULTRAFILTRATE ASSAY

SUBJECT	POSITION	TIME	FILTRATE VASOCONSTRICTOR ACTIVITY
H. E. H.	Lying (80 min.)	80	Equivalent to normal saline; less than epinephrine 0.01 μg per liter
	Standing (40 min.)	120	Between epinephrine 0.01 and 0.1 μg per liter
J. L. C.	Lying (90 min.)	90	Equivalent to normal saline; less than epinephrine 0.01 μg per liter
	Standing (40 min.)	135	Equivalent to epinephrine 0.1 μg per liter
D. A. N.	Lying (86 min.)	80	Equivalent to normal saline; less than epinephrine 0.01 μg per liter
	Standing (35 min.)	121	Slightly greater activity than epinephrine 0.01 μg per liter

This table gives the data showing the appearance of a circulating vasoconstrictor substance when a normal subject assumes the erect position from the horizontal. The assay was kindly done by Armin and Grant.

The first blood sample withdrawn, while the subject was lying horizontally and quietly, revealed no detectable vasoconstrictor activity (less than the epinephrine 0.01 μg per liter); the second blood specimen, withdrawn after 40 minutes of quiet standing, revealed a vasoconstrictor activity of the magnitude shown between an epinephrine equivalent of 0.01 and 0.1 μg per liter. These data are shown in Table II.

DISCUSSION

We have shown that when a normal subject assumes the erect position from the horizontal, there is a decrease in the renal excretion of water, sodium,

potassium, and chloride. At the same time, detectable vasoconstrictor activity (substance unknown) in the subject's circulating blood appears. In other conditions reported in which there is a proportionately greater decrease in renal blood flow than in glomerular filtration rate, with a resulting rise in the filtration fraction, there is a decrease in the excretion of sodium by the kidney.[5, 6] Nickel and associates showed this to be true in their *l*-norepinephrine infusion experiments. If a vasoconstrictor substance like *l*-norepinephrine can initiate a decrease in sodium and potassium excretion by causing a relative ischemia of the renal tubules, the decrease in sodium, chloride, and potassium excretion on quiet standing might be due to the appearance of a vasoconstrictor substance in the blood. That this vasoconstrictor substance might be *l*-norepinephrine is suggested by the findings of Von Euler and co-workers.[7] They found that normal subjects, placed on a tilting table at an angle of 75 degrees for 3 or 4 hours, show a considerable increase in the urinary output of norepinephrine in comparison with the corresponding excretion in the recumbent position.

SUMMARY

It has been found that vasoconstrictor activity appears in the circulating blood on assuming the upright position from the recumbent. The hypothesis is formulated that this vasoconstrictor activity in the blood might be, through changes in renal blood flow, the cause of the decrease in renal excretion of sodium, potassium, and chloride which occurs on quiet standing. The experiments of Von Euler are discussed as evidence that this vasoconstrictor substance might be norepinephrine.

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WHOLE BLOOD RIBOFLAVIN LEVELS IN HEALTHY INDIVIDUALS AND IN PATIENTS MANIFESTING VARIOUS BLOOD DYSCRASIAS

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SINCE the introduction of the microbiologic assay for riboflavin in 1939 by Snell and Strong,[1] it has been utilized for the measurement of this vitamin in a multitude of biologic specimens. At the present time, however, there is no substantial agreement among authors concerning the concentration of riboflavin in human blood samples. This discrepancy may be attributed to the differences in the techniques which have been employed for measurement. The early workers[2, 3] applied microbiologic assay of riboflavin to blood and used hemolyzed whole blood samples. The limitations of utilizing this method without an extraction prior to assay have been clearly pointed out by Bradford and Coke.[4] The values obtained by these latter workers for a series of 8 normal individuals had a mean of 21.2 μg per cent, which is roughly half that found by the earlier investigators. Using a fluorometric method of analysis, other workers[5-7] have reported mean riboflavin levels of 5.7, 6.7, and 12.2 μg per cent. These values are all lower than those obtained using the microbiologic assay method.

The lack of agreement concerning whole blood riboflavin levels has led us to reinvestigate its measurement microbiologically with *Lactobacillus casei*. It is well documented that microbiologic measurement reflects total riboflavin, as the bacteria respond in an equivalent manner to the free vitamin and to its coenzymes, riboflavin phosphate (FMN) and flavin-adenine dinucleotide (FAD). Throughout this paper our expression of riboflavin concentration is in the sense of this total active material. With a modification of the method of Bradford and Coke,[4] 70 normal bloods have been studied. The results of these determinations together with an analysis of some of the factors influencing riboflavin concentration in blood are presented. Included in this report are studies on patients with leukemia, anemia, and other hematologic disorders.

METHODS

Oxalated samples of venous blood are drawn and are refrigerated in brown bottles prior to assay. Samples could be stored in this manner for 14 to 17 days without significant

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variation in the observed riboflavin level, but beyond 20 days the reproducibility on successive determinations was decreased. In the data presented here no sample was stored for more than 12 days prior to assay.

The assay procedure is a modification of that described by Bradford and Coke.[4] One milliliter of whole blood is mixed with 4 ml. of 0.1 N HCl in a 16 by 150 mm. test tube The tubes are stoppered with cotton plugs and autoclaved at 15 pounds steam pressure (121° C.) for 15 minutes. This mixture is cooled to room temperature and then neutralized with 1 N NaOH (0.4 ml.). The pH is adjusted to 4.5 with approximately 5 drops of 2 N acetic acid (narrow range pH paper). The tubes are centrifuged at 800 g for 20 minutes, and the supernatant is decanted into a small beaker. The precipitate is resuspended twice in 1 ml. of water (pH 4.5), and centrifugation is repeated. The combined supernatants are adjusted to pH 6.7 (point of heaviest precipitation) with 1 N NaOH and then filtered through Whatman No. 42 paper into 10 ml. volumetric flasks. The beakers and the filter papers are washed with small portions of water (pH 6.7), and the filtrates are diluted to volume.

Bacterimetry is conducted by established procedures[8] using Difco Riboflavin Assay Medium. To each of the assay tubes, 2 ml. of the extract is added. The tubes are set up in triplicate with one being reserved as an extract blank. With this procedure, the most useful set of standards is 0.000, 0.010, 0.015, 0.020, 0.030, 0.040, 0.050, 0.070, and 0.100 μg riboflavin per tube (10 ml.). An uninoculated medium blank is also included for control purposes. Each tube is mixed, stoppered, and autoclaved at 15 pounds pressure for 10 minutes.

After autoclaving, the tubes are allowed to cool to room temperature. For convenience the tubes are most often placed in the refrigerator and inoculated the following morning. Except for the extract and medium blanks, the tubes are inoculated with a saline suspension of *Lactobacillus casei* and are incubated at 37° C. for 72 hours. Using a Beckman pH meter, the acid produced is titrated with approximately 0.045 N NaOH to pH 6.7. The contents of the tubes along with a 10 ml. wash are agitated with a magnetic stirrer during titration. The standard curve is obtained by a plot of the milliliters of NaOH vs. log concentration of riboflavin per unit volume. The titration for each assay tube is corrected by the blank values, read from the standard curve, and calculated to μg per cent riboflavin using a suitable dilution factor.

When riboflavin was added to whole blood just prior to extraction or to the extracts prior to assay, it was quantitatively recovered by the technique just described. In agreement with the findings of others[9-11] incorporation of an ether extraction into the procedure in addition to filtration did not alter the values obtained and was not routinely used.

This method has largely obviated the discrepancy between values obtained with different sample sizes; e.g., no significant difference was found in the riboflavin concentration of a blood sample when assaying 0.2 ml. of blood (2.0 ml. of extract) as compared to 0.3 ml. of blood (3.0 ml. of extract). A slight bias in favor of elevated values was introduced, however, by going to still smaller samples. From a scatter diagram and a least squares plot, an empirical relationship has been derived, equating the value expected from assay of 0.1 ml. (Y) and 0.2 ml. (X) of blood: $Y = 1.17\ X - 2.0$.

RESULTS

The 70 normal individuals used in this study were healthy laboratory workers and blood bank donors, and the results of whole blood riboflavin assays on these individuals were analyzed for differences that could be attributed to the age of the donor, the sex of the donor, or the number of hours fasting (Table I). No significant differences were found when the group was subdivided with respect to age or sex. However, the mean for the group that had been fasting less than 8 hours was found to be slightly elevated as compared to the group

that had been fasting over 8 hours; although the difference was small, it was found to be statistically significant at the 5 per cent probability level ($t = 1.958$; d.f. $= 35$; $P = 0.05$).

TABLE I. FACTORS ANALYZED FOR EFFECT ON WHOLE BLOOD RIBOFLAVIN LEVEL IN NORMAL INDIVIDUALS

CLASSIFICATION	NO. OF INDIVIDUALS	μG RIBOFLAVIN/100 ML. BLOOD	
		MEAN	STANDARD ERROR
Age			
18-29	26	15.6	0.56
30-39	26	17.8	0.65
40-52	17	16.9	0.88
Race			
Negro	2	19.3, 14.0	---
white	67	16.7	0.41
Sex			
female	11	15.7	1.03
male	58	16.9	0.41
Hours fasting			
<8	43	17.4	0.51
>8	24	15.5	0.65
Total	70	16.7	0.36

TABLE II. WHOLE BLOOD RIBOFLAVIN LEVELS IN LEUKEMIC PATIENTS BEFORE INSTITUTION OF THERAPY

DIAGNOSIS	NO. OF PATIENTS	NO. PATIENTS WITH ELEVATED RIBOFLAVIN*	μG RIBOFLAVIN/100 ML. BLOOD		P†
			MEAN	S. E.	
Normal	43	1	17.4	0.51	--
All leukemics	34	10	24.5	2.87	<0.01
Acute					
lymphocytic	12	3	20.8	2.02	0.05
myelocytic	5	2	35.7	14.70	<0.01
monocytic	3	0	19.7	2.17	0.20-0.30
Chronic					
lymphocytic	7	1	20.5	4.32	0.10-0.20
myelocytic	7	4	28.9	6.95	<0.01

*That is, >24.0 μg per cent; compare with Fig. 1.

†Probability that the observed deviation of the mean from that for the normal group could be due to chance alone.

Sixty-five patients with the following hematologic disorders were studied: leukemia, anemia, polycythemia vera, idiopathic thrombocytopenic purpura, Hodgkin's disease, multiple myeloma, and lupus erythematosis. The majority of these, however, were patients with either leukemia or anemia. No abnormalities of whole blood riboflavin concentration were found in any of the pathologic groups except among untreated leukemic patients. The distribution of values for the latter as compared to the distribution for the normal individuals is presented in Fig. 1. The feature of interest to be observed is that 30 per cent of the leukemic patients exhibit riboflavin levels higher than 24 μg per cent which, with the exception of one individual, is the upper limit observed in the normal series. Further subdividing the patients with leukemia according to differential diagnosis, it was noted that the elevated values were manifest primarily in the acute and chronic myelocytic forms of the disease. The deviations of the means for these two groups from the mean for the normals are seen

by reference to Table II to be significantly different at the 1 per cent probability level. These comparisons were made with the group of normal individuals fasting less than 8 hours, as they correspond most closely to the fasting state of the patients. It is to be noted that 50 per cent of the patients with myelocytic leukemia showed elevated riboflavin values, ranging for the most part from 30 to 45 μg per cent. One patient with the chronic condition, however, had a riboflavin level of 65 μg per cent; the level for one with the acute form was 93 μg per cent.

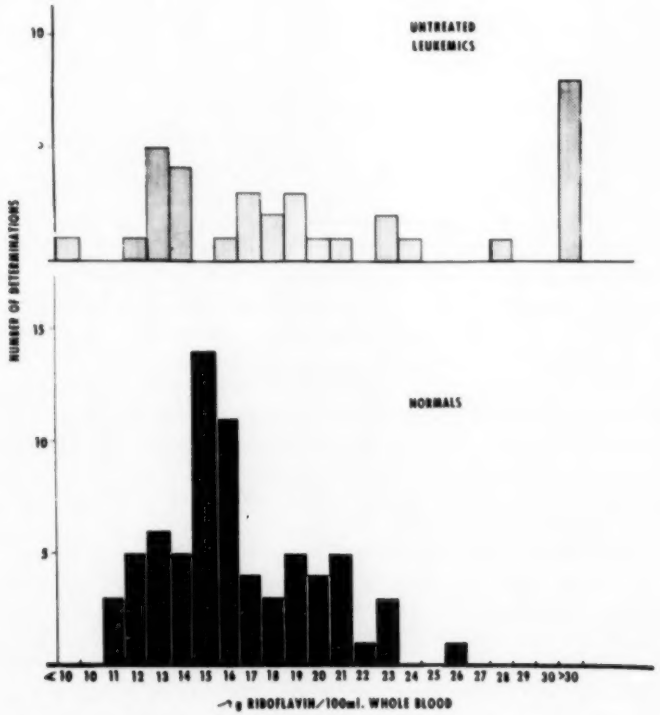

Fig. 1.—Distribution of riboflavin levels in the blood of normal and leukemic individuals.

TABLE III. TIME LAPSE FOR GIVEN CHANGES IN BLOOD RIBOFLAVIN LEVEL FOLLOWING INTRAVENOUS INJECTION

△ CONCENTRATION (μG/100 ML.)	△ TIME (MIN.)			
	NORMAL		LEUKEMIC	
	M. G.	M. M.*	S. E.†	B. H.‡
50-40	--	4	11	66
40-30	10	10	19	273
30-20	32	32	37	--

*Received riboflavin phosphate; others received free riboflavin.
†Acute monocytic leukemia.
‡Acute lymphocytic leukemia.

The rate of disappearance of riboflavin activity from peripheral blood following intravenous injection of 25 to 50 mg. was determined for 2 normal and 2 leukemic individuals. In Table III the time required for given changes in concentration is recorded. It is interesting that patient B. H., who exhibited a low removal rate, also had an increased initial riboflavin level (25.5 μg per cent).

DISCUSSION

The values reported here for riboflavin activity in normal whole blood are smaller than those found by Bradford and Coke.[4] The mean values for the two series of normal individuals are 15.9 and 21.2 μg per cent. Two variations in procedure may account for the observed differences. The standard curve which has been used in this study has a larger number of standards in the range of lower riboflavin concentrations, and it is believed to have given increased control over the assay. More importantly, perhaps, has been the inclusion, as a means of removing fatty substances, of a filtration step in the extraction procedure. As pointed out by others,[9-11] fatty acids may interfere with the microbiologic determination of riboflavin because of their effect in stimulating the growth of Lactobacilli at low concentrations and inhibiting their growth at elevated concentrations.

Fluorometric determination of riboflavin in blood has given uniformly lower values than have been found microbiologically. Burch, Bessey, and Lowry,[7] using a direct fluorometric determination on blood extracts, report a value of 12.2 μg per cent. Kerppola,[6] applying the method of Fujita and Matsuura[5] in which riboflavin is converted by photolysis to lumiflavin, found a mean riboflavin concentration of 6.7 μg per cent for 60 normal individuals. His highest value of 10.4 μg per cent is lower than the lowest value (11.0 μg per cent) in our own normal series. Further work must yet be conducted to resolve the differences obtained by the two assay methods.

Of the hematologic disorders studied, only the group of untreated cases of leukemia showed any abnormality. Within this group the elevation was most marked in the myelocytic form of the disease. Kerppola[6] also found an increase in myelocytic leukemia (mean = 13.9 μg per cent). Despite the differences in absolute values in his series as compared to our own, it is significant that both methods lead to the same conclusion. It may be worth noting that in studies on serum, it is also in patients with myelocytic leukemia that another of the B vitamins, namely vitamin B_{12}, has been found elevated.[12, 13]

Statistical analysis has revealed poor correlation between whole blood riboflavin activity and either erythrocyte or leukocyte count (correlation coefficient, r, = 0.383 and 0.291, respectively). The one exceptional condition in which a high degree of correlation has been observed is for those bloods in which the leukocyte count exceeds 100,000 ($r = 0.935$; $t = 5.870$; d.f. $= 5$; $P < 0.01$).

Although riboflavin disappearance rates have been determined for only a small group of individuals, one is tempted to speculate on the possible significance of the data (Table III). It is probably fortuitous that the rates for the two normal individuals were identical, but, as one received riboflavin and the other

riboflavin phosphate, it may indicate that the free vitamin and the coenzyme are disposed of similarly following intravenous injection. That patient B. H. showed a reduced disappearance rate coupled with an elevated blood level may point to an abnormal process affecting this vitamin.

SUMMARY

Several improvements in the microbiologic assay of riboflavin in human whole blood are presented. A mean riboflavin concentration of 15.9 μg per cent was found for a series of 70 normal individuals. No variation with respect to age or sex was observed, although a small but significant variation with respect to fasting state was noted. In a variety of hematologic disorders studied, abnormalities were found only among patients with untreated leukemia. Elevations were noted particularly in the myelocytic form of the disease. Riboflavin concentration correlates with white cell count only when it becomes 100,000 or greater. The rates of disappearance of riboflavin from the blood following intravenous injection have been determined for two normal and two leukemic individuals.

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THE TURNOVER OF FLUID IN THE DOG'S PLEURAL CAVITY

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INTRODUCTION

VON Recklinghausen[1] and Dybkowsky[2] considered the serous cavities as part of the lymphatic system and the absorption of fluid from these cavities as occurring into the serosal lymph vessels. Dybkowsky also recognized that respiratory movements might provide the force needed to move fluids in the pleural lymphatics. This theory of lymphatic absorption was disputed by Starling and Tubby,[3] who found that hypotonic solutions of sodium chloride, glucose, and low molecular weight dyes such as methylene blue and indigo carmine were rapidly removed directly into the blood stream. Later, Leathes and Starling[4] concluded that the rates of absorption of various substances from the pleural cavity could be understood satisfactorily if the pleural membranes were a size-selective barrier controlling entry into the circulation along diffusional or osmotic gradients. The relative rates of removal of substances from the pleural cavity would thus be due to the ease with which they could penetrate the membrane. Leathes and Starling conceded that the lymphatics might play a part in the removal of isotonic fluid from the pleural cavity but offered no proof of this.

Courtice and Simmonds[5] re-examined the whole question of lymphatic absorption from the pleural cavity by introducing fluid containing a protein antigenically distinguishable from the animal's own protein or a protein labeled with the dye T1824. They showed that in the cat, labeled protein was removed mainly or wholly by the lymphatics. When the right lymph duct and the thoracic duct were cannulated, no dye reached the circulation; further, the dye that left the pleural cavity could be accounted for in the lymph in these channels. Courtice and Simmonds[6] emphasized in a later review that the failure to distinguish between the movement of small molecules directly into the blood stream through the pleural capillaries, and the bulk transfer of large molecules, such as protein, solely by the pleural lymphatics had led to erroneous estimates of the rates of absorption from the pleural cavity.

Starling and Tubby[3] showed that hypertonic solutions of glucose or saline placed in the pleural cavity consistently led to large increases in the volume of pleural fluid. Pinner and Moerke[7] found in rabbits that the normal pleura was permeable in either direction to many of the constituents of the blood.

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The present work is an attempt to study quantitatively the factors responsible for the movement of fluid, solutes, and cells into and out of the pleural cavity.

METHODS

Collection of Stored Serum.—When stored serum was required the dog was bled into 250 ml. glass centrifuge buckets, the blood defibrinated with a glass rod and centrifuged. The serum was separated, Seitz-filtered, and stored at 4° C. for periods up to 3 weeks before being used.

Para-aminohippurate Estimation.—This was estimated by the method of Smith and associates[8] and read at 540 mμ.

Protein Estimation.—Total protein was measured by the biuret method of Robinson and Hogden.[9] Samples containing T1824 needed decolorization. In this case after the 3 per cent NaOH had been added to the sample, about 50 mg. of Raney nickel-aluminum alloy was also added; the sample was left at room temperature for at least 3 hours for the catalytic hydrogenation of the T1824 to proceed to completion. The solution was then filtered, and $CuSO_4$ solution added to an aliquot. The absorption was read at 560 mμ.

Intrapleural Pressure Measurements.—The intrapleural catheter was connected to a Sanborn electromanometer and the pressures recorded on a Viso-Cardiette. The pressures were measured with the dogs prone, and the zero reference level was taken to be midway between sternum and spine. Care was taken that no bubbles remained in the system.

RESULTS

Fluid Movement After Intrapleural Injection of Fresh Heparinized Plasma in Dogs at Rest.—Using the method previously described[10] the turnover of fluid in the dog's pleural space was measured. Fig. 1 shows the results of a typical experiment in an anesthetized dog at rest. The lymphatic removal averaged 0.62 ml./Kg. hr. and the nonlymphatic fluid entry was 0.55 ml./Kg. hr. Table I shows the results obtained in 5 similar experiments. The lymphatic flow

TABLE I. AVERAGE RATES OF FLOW OF FLUID INTO AND OUT OF THE PLEURAL CAVITY OF RESTING UNANESTHETIZED DOGS AFTER INJECTION OF FRESH HEPARINIZED PLASMA

EXPERIMENT	WEIGHT OF DOG	LYMPHATIC FLOW (ML./KG. HR.)	NONLYMPHATIC FLOW (ML./KG. HR.)	RATE OF CHANGE IN VOLUME OF PLEURAL FLUID (ML./KG. HR.)	AVERAGE VOLUME OF PLEURAL FLUID (ML./KG.)
68	19.1	0.65	0.54	−0.11	10.7
69	15.9	0.60	0.60	0.00	12.1
71	15.9	0.79	0.47	−0.32	4.1
73	16.8	0.48	0.42	−0.06	15.5
74	14.5	0.35	0.33	−0.02	10.3
Mean	16.4	0.57	0.47	−0.10	11.75
Standard error of mean		±0.082	±0.053	±0.065	

averaged 0.57 ± 0.082 (S.E. of mean) ml./Kg. hr., and the extralymphatic flow, which in every case was from the blood to pleural fluid, averaged 0.47 ± 0.053 (S.E. of mean) ml./Kg. hr. Thus, there was a net fall in pleural fluid volume of 0.10 ml./Kg. hr. equivalent to 48 ml./24 hr. for a 20 kilogram dog. However, there was rarely any fluid left 24 hours after the beginning of the experiments. In some cases the rate of fluid entry declined after several hours.

This would lead to a speeding up of the net rate of removal. In addition, the dogs were very quiet during the experiment and tended to compensate for their boredom by intense activity when returned to the animal house. It will be shown later that activity accelerates the lymphatic flow. These two factors may account for the disappearance of most of the fluid during the night.

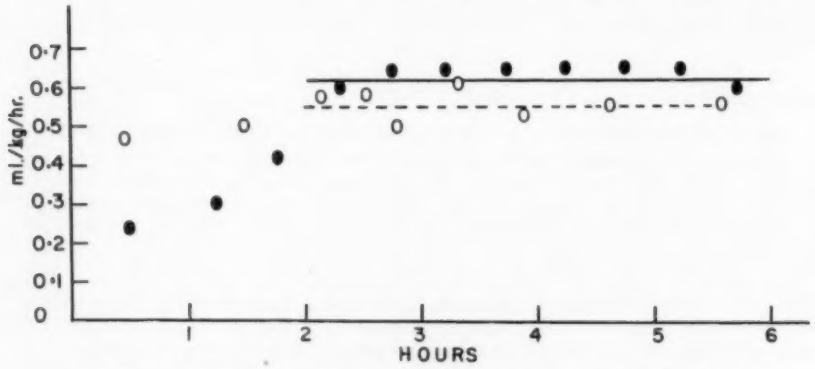

Fig. 1.—Lymphatic and nonlymphatic flow in an unanesthetized dog at rest.

Key: ●——● = lymphatic flow $\left(\frac{dV_L}{dt}\right)$; ○- - - -○ = nonlymphatic flow $\left(\frac{dV_{NL}}{dt}\right)$; ordinates = ml./Kg. hr.; abscissas = time in hours.

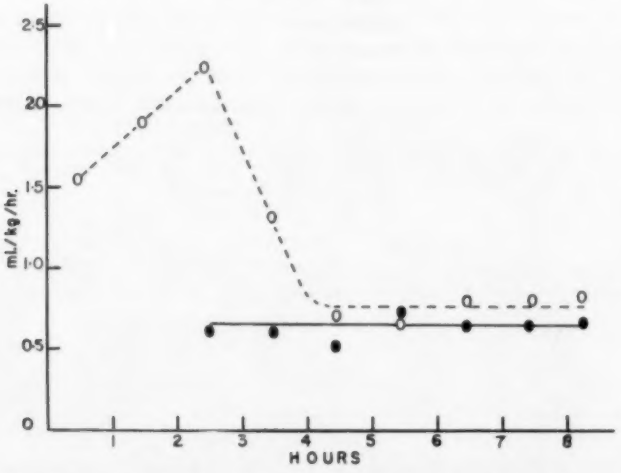

Fig. 2.—Resting unanesthetized dog, 20.2 kilograms. Lymphatic flow and nonlymphatic flow. At zero time 140 ml. of stored serum was injected into the right pleural cavity. A high rate of fluid entry into the pleural cavity was present for the first 4 hours reaching a peak of 2.25 ml./Kg. hr. in the third hour.

Key: ●——● = lymphatic flow; ○- - - -○ = nonlymphatic flow; ordinates = ml./Kg. hr.; abscissas = time in hours.

When these experiments were started it was not appreciated that it was important to use fresh plasma collected without contact with water-wettable

surfaces. In these early experiments plasma or serum was stored for days or weeks before use. Almost invariably this resulted in a high rate of fluid entry into the pleural cavity for several hours. The experiment illustrated in Fig. 2 shows the effect of injecting into the pleural cavity 140 ml. of serum stored at 4° C. for 4 weeks. The rate of fluid entry reached a peak of 2.25 ml./Kg. hr. at 2½ hours and even after 8 hours the rate of entry exceeded the lymphatic flow, and thus the volume of pleural fluid was still increasing. The injection of stored serum intrapleurally affected only the transfer of extralymphatic fluid and not the lymphatic flow. In more than half the experiments there was an increase in the volume of pleural fluid due to the initial high rate of fluid entry.

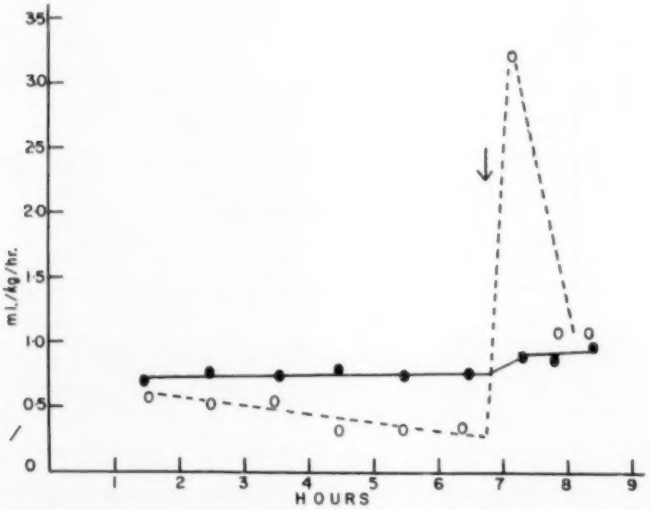

Fig. 3.—Resting unanesthetized dog, 15.9 kilograms. One hundred and twenty milliliters of fresh heparinized plasma labeled with I^{131} albumin injected into the right pleural cavity at zero time. At the point indicated by the arrow, 20 ml. of stored serum was injected into the pleural cavity. A large nonlymphatic flow of 0.5 to 3.2 ml./Kg. hr. occurred.

Key: ●——● = lymphatic flow; ○- - - -○ = nonlymphatic flow; ordinates = ml./Kg. hr.; abscissas = time in hours.

Stored serum could produce a similar increase in fluid entry if introduced in the course of an experiment initiated with fresh heparinized plasma (Fig. 3). In this case, 20 ml. of serum that had been stored for 6 weeks at 4° C. was injected intrapleurally after a control period of 6¾ hours. The fluid entry increased from 0.3 ml./Kg. hr. to 3.2 ml./Kg. hr., and in this experiment there was also a small increase in lymphatic flow.

Courtice and Simmonds[5] had noticed a similar effect of injecting homologous serum or plasma into the rabbit's pleural cavity, and found that this effect could be reduced or eliminated by storage of the serum for 4 weeks. This was not the case with dog serum and indeed the effect became more pronounced with storage up to 6 weeks. A number of drugs (pyranisamine, chlorprophenpyridamine, adrenalin, 5-hydroxytryptamine, lysergic diethylamide

[LSD], procaine, and lignocaine) were tried either locally or systemically to see if they would antagonize the effect of stored serum on the fluid entry, but without success.

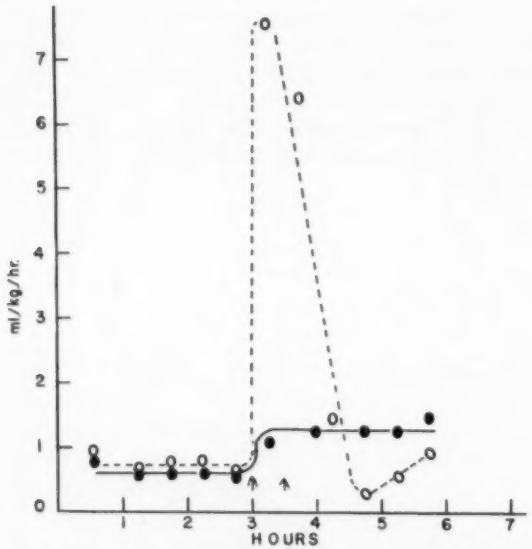

Fig. 4.—Resting unanesthetized dog, 16.5 kilograms. The effect of intrapleural injection of histamine. One-hundred and fifty milliliters of fresh heparinized plasma was injected into the pleural cavity and after a control period of 3 hours, 18 μg of histamine was injected into the pleural fluid; this was followed by a similar dose 30 minutes later as indicated by the arrows. A large increase in nonlymphatic flow movement occurred reaching a peak of 7.6 ml./Kg. hr. in the first 30 min. The pleural fluid volume increased by 6.1 ml./Kg. in the first hour.

Key: ●——● = lymphatic flow; ○- - - -○ = nonlymphatic flow; ordinates = ml./Kg. hr.; abscissas = time in hours.

Histamine and Histamine Liberators.—Effects similar to those obtained with stored serum were found when small doses of histamine were injected into the pleural cavity. In Fig. 4 the injection of two doses of 18 μg of histamine increased the fluid entry tenfold from 0.7 ml./Kg. hr. to 7.6 ml./Kg. hr., and over a period of one hour the volume of pleural fluid increased by 100 ml. There was also a moderate increase in the lymphatic flow. An effect similar in character was produced by the histamine-liberating drugs diaminodecane, 48/80, and dihydroergotamine when injected into the pleural cavity. The effects of both histamine and histamine liberators were completely abolished by small doses of pyranisamine given intrapleurally (0.5 mg.) or intravenously (2 mg./Kg.).

Isotonic Saline in the Pleural Cavity.—After the injection of 10 to 15 ml./Kg. of 0.9 per cent NaCl, to which had been added trace amounts of iodinated bovine albumin or T1824 (with or without 500 mg. of bovine albumin as a carrier for the dye), two basic types of response were seen. Seventeen dogs were studied; in 12 of the dogs used, the extralymphatic flow was small but in the direction of blood to pleural cavity. In a typical example, the

extralymphatic flow was only 0.12 ml./Kg. hr. In two experiments the extralymphatic flow was reversed so that fluid was being removed directly into the blood stream at a maximum rate of 0.1 ml./Kg. hr.

The less common response, seen in 3 dogs, is illustrated in Fig. 5. In this experiment there was initially a small fluid entry which gradually built up to a peak after 4½ hours at which time the fluid entry was 2.65 ml./Kg. hr. The effect gradually declined but had not entirely disappeared 8 hours after the start of the experiment, and, as the rate of fluid entry was still higher than the lymphatic flow, the volume of pleural fluid was still increasing.

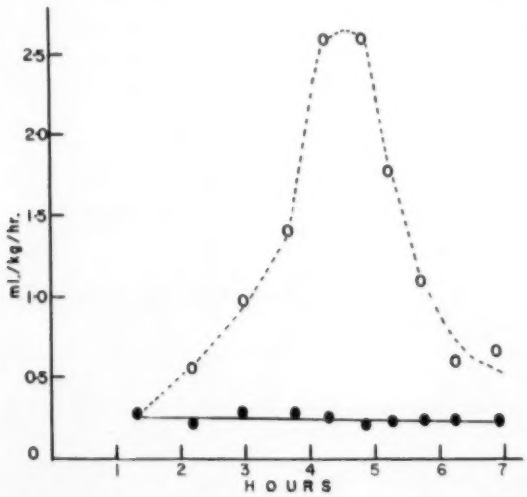

Fig. 5.—Resting unanesthetized dog, 24.1 kilograms. One hundred and fifty milliliters of 0.9 per cent NaCl containing 38.64 mg. T1824 was injected into the right pleural cavity. Initially there was a small nonlymphatic flow which increased to a maximum of 2.65 ml./Kg. hr. at 4½ hours and then gradually declined.

Key: ●——● = lymphatic flow; ○- - - -○ = nonlymphatic flow; ordinates = milliliters per kilogram hour; abscissas = time in hours.

The lymphatic flow in both groups of dogs after injecting isotonic saline into the pleural cavity was 0.49 ± 0.09 (S.E. of mean) ml./Kg. hr. which was not significantly different from the lymphatic flow in the dogs in which fresh heparinized plasma had been used.

It was also found that only small amounts of protein entered the pleural cavity in the first type of response in which there was a small rate of fluid entry after the injection of isotonic saline. On the average the rate of protein entry was 20.0 mg./Kg. hr. This was very similar to the rate of protein entry when fresh heparinized plasma has been injected into the pleural cavity. In the second type of response to the injection of isotonic saline in the pleural cavity, however, the protein entered about 5 times as rapidly, and in the 3 experiments the mean rate of protein entry was 93 mg./Kg. hr. at its peak.

The two types of response can be correlated with the rate of loss of intravenous label from the blood plasma. This rate of loss of protein from the

plasma into the extravascular space is a measure of total capillary permeability to protein and is constant in any individual dog studied at different times with or without an injection of fluid into the pleural cavity. This has also been the experience of Price and Longmore.[11] In the present experiments, when intrapleural injection of isotonic saline resulted in a large extralymphatic flow into the pleural cavity and a high rate of entry of protein, the loss of albumin from the blood was rapid (mean: 10.8 per cent of the circulating albumin per hour); when only a small extralymphatic flow resulted, the rate of loss of albumin from the blood was smaller (mean: 5.9 per cent per hour).

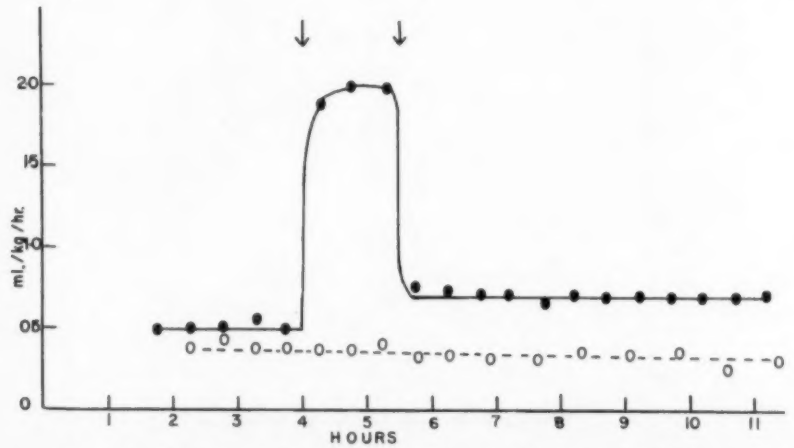

Fig. 6.—Resting unanesthetized dog, 16.8 kilograms. Fresh heparinized plasma, 247 ml., labeled with I^{131} albumin was injected into the right pleural cavity. Effect of hyperventilation due to inhalation of 7 per cent CO_2. The period of inhalation is indicated by the arrows. There was a considerable increase in lymphatic flow during the period of hyperventilation. The lymphatic flow remained elevated above the control level after the cessation of hyperventilation.

Key: ●——● = lymphatic flow; ○- - - -○ = nonlymphatic flow; ordinates = milliliters per kilogram hour; abscissas = time in hours.

Effect of Changed Respiratory Activity.—In all 11 dogs studied, hyperventilation induced by the administration of 3 to 7 per cent CO_2 in air produced a considerable increase in the lymphatic flow without effect on the extralymphatic fluid movement. In the experiment illustrated in Fig. 6, the inhalation of 7 per cent CO_2 in air increased the lymphatic flow from 0.5 ml./Kg. hr. to 2.1 ml./Kg. hr. When the dog again breathed room air the lymphatic flow remained elevated above the control level. This persistence of an elevated lymphatic flow after removal of the exciting cause has been found in other experiments in which an increased lymphatic flow occurred.

The lymphatic flow was reduced by depression of the respiratory rate with anesthesia. Light pentobarbitone anesthesia depressed the lymphatic flow in 3 dogs from 0.63 ml./Kg. hr. to 0.29 ml./Kg. hr. In dogs anesthetized from the start of the experiment the mean rate was as low as 0.13 ml./Kg. hr. The significance of this difference is discussed later. Anesthesia also depressed, and in one experiment stopped, the extralymphatic flow.

The Effect of Changes in Intrapleural Pressure.—Since the movement of fluid across the pleural capillaries must be dependent on hydrostatic pressure gradients, it would be expected that the intrapleural pressure would have an important influence on the extralymphatic fluid movement. This is borne out in the experiment illustrated in Fig. 7. In this experiment stored serum had been injected into the pleural cavity 16 hours previously, and the label (T1824) was added at zero time. After a control period of 3½ hours the extralymphatic fluid entry was steady at 0.3 ml./Kg. hr., and the intrapleural pressure was –3.5 cm. H_2O (referred to atmospheric pressure). The mean intrapleural pressure was raised to +2 cm. H_2O by the injection of an extra 320 ml. of stored serum. The intrapleural pressure gradually decreased over the next 4½ hours to –3.5 cm. H_2O. The extralymphatic fluid entry from the blood into the pleural cavity ceased when the intrapleural pressure became positive.

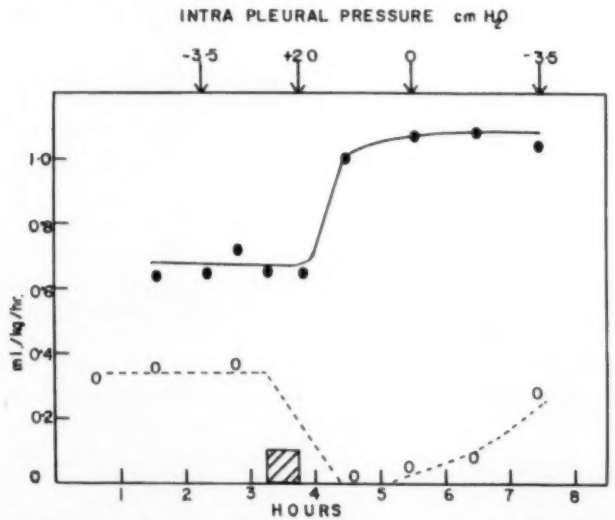

Fig. 7.—Resting unanesthetized dog, 22.3 kilograms. One hundred and fifty milliliters of stored serum was injected into the pleural cavity 17 hours before the start of the experiment. Sixty-five milligrams of T1824 was injected at zero time. The pleural volume at this time was 128 ml. During the period indicated by the cross-hatched block a further 320 ml. of stored serum was injected, raising the intrapleural pressure from –3.5 cm. H_2O to +2 cm. H_2O. The nonlymphatic flow ceased and gradually returned as the negative intrapleural pressure was re-established. There was some increase in lymphatic flow.

Key: ●——● = lymphatic flow; ○- - - -○ = nonlymphatic flow; ordinates = milliliters per kilogram hour; lower abscissa = time in hours; upper abscissa = intrapleural pressure cm. H_2O.

As the negative pressure became re-established, the rate of fluid entry increased until at 7½ hours it had almost returned to the initial rate. There was maintained increase in the lymphatic flow following the injection of serum, probably due to the increased respiratory effort secondary to collapse of the lung.

A decrease in intrapleural pressure was produced by negative pressure breathing in anesthetized dogs. In the experiment illustrated in Fig. 8 the

mean intratracheal pressure was reduced to –10 cm. H_2O, leading to a fall in the mean intrapleural pressure from –5 cm. to –16 cm. H_2O. The rate of fluid entry into the pleural cavity was doubled. This rate fell immediately when the intratracheal and intrapleural pressures were restored to normal.

Initially in this experiment the lymphatic flow was low due to the effects of anesthesia. During the period of negative pressure breathing a marked increase in lymphatic flow accompanied the increase in rate and depth of respiration.

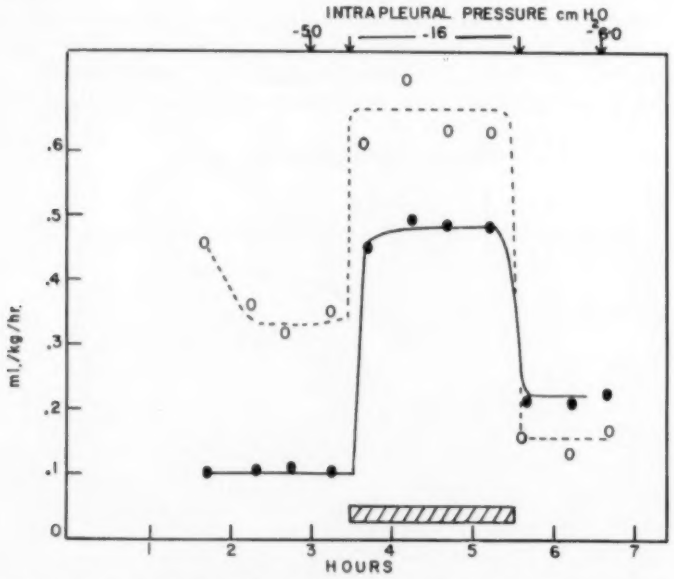

Fig. 8.—A dog weighing 20 Kg. was anesthetized with 50 mg. pentobarbitone per kilogram of weight. Two hundred and fifty-five milliliters of plasma labeled with I^{131} albumin was injected into the pleural cavity. For the time between 3½ and 5½ hours, as shown by the cross-hatched area, the animal's trachea was connected to a negative pressure system which caused a fall of mean intrapleural pressure from –5 cm. H_2O. Both lymphatic and nonlymphatic flows were increased.

Key: ●——● = lymphatic flow; ○- - - -○ = nonlymphatic flow; ordinates = milliliters per kilogram hour; lower abscissa = time in hours; upper abscissa = intrapleural pressure in cm. H_2O.

The Removal of Low Molecular Weight Substances From the Pleural Cavity.—During the course of an experiment in a resting dog into which either serum or fresh plasma had been injected intrapleurally, *p*-aminohippurate or tracer amounts of Na^{22} as $Na^{22}Cl$ was injected into the fluid present in the pleural cavity. It was found that the concentrations of these substances fell exponentially with time. In one experiment with Na^{22} in a dog weighing 19.5 kilograms, the exponential rate constant was 1.46/hr.; the mean volume of pleural fluid during this time was 480 ml., and hence the clearance rate was 700 ml. plasma per hour (i.e., 480 × 1.46). Assuming that no appreciable amount of Na^{22} penetrates the erythrocytes, this gives a whole blood clearance

of 71 ml./Kg. hr. in this dog. In another experiment when *p*-aminohippurate was used, the rate constant was 1.14/hr., and the plasma clearance 230 ml./hr. The whole blood clearance was 29 ml./Kg. hr.

It had been found previously[12] that when the pleural fluid was made hypertonic by the injection of concentrated NaCl solution, water entered the pleural cavity at a rate corresponding to a clearance of water from the blood of 81 ml./Kg. hr. (exponential rate constant 4.2/hr.).

The similar magnitude of the transfer rates of Na^{22} and water suggest that they were limited primarily by the pleural blood flow. We can thus set a lower limit for the pleural blood flow of 70 to 80 ml./Kg. hr. The normal fluid entry at rest represents less than 1 per cent of the volume of blood that traverses the pleural vessels, and at the maximum rate of entry it may reach 10 per cent of the pleural blood flow.

The slower disposal of *p*-aminohippurate is presumably due to this, being in part limited, not by blood flow, but by restricted diffusion through the tissues.

The rapidity with which small molecules are removed from pleural fluid makes it clear that they are absorbed directly into the pleural circulation and, unlike the proteins, are not limited by the rate of lymphatic drainage.

The Protein and Cell Concentrations in Pleural Fluid.—

a. Naturally-occurring fluid: The amount of naturally-occurring pleural fluid was determined by two methods. Six dogs were killed by intravenous Nembutal, and the chest was opened carefully to avoid contamination. The geometric mean of the volumes of fluid found was 1.3 ml. The other method was by calculation, noting the dilution of the injected label immediately after the intrapleural injection of a known volume of fluid. In 16 experiments where this was done the geometric mean of the amount of naturally-occurring fluid present was 2.7 ml. The difference between the two methods is presumably due to the capillary layer of fluid moistening the pleural surfaces that cannot be removed by aspiration.

TABLE II. NATURALLY OCCURRING PLEURAL FLUID IN THE DOG

DOG	VOL. OF FLUID IN ML. (BOTH SIDES)	PROTEIN (GM./100 ML.)	W.B.C./MM.^{3}	R.B.C./MM.^{3}	CIRCULATING PLASMA PROTEIN (GM./100 ML.)
1	7.5	1.2	2,600	5,000	—
2	0.6	2.1	9,700	30,000	6.65
3	0.3	0.71	2,200	30,000	7.8
4	0.2	3.5	6,000		7.1
5	0.3	0.5	850	450	6.8
6	15.0	2.4	1,800	6,000	7.7
Mean	1.3 (geometric mean)	1.39% 0.8-2.5 95% limits	2,820 1,330-6,020 95% limits	6,300 1,090-36,300 95% limits	7.2 ± 0.48 (S.D.)

The protein concentration of the naturally-occurring pleural fluid obtained was 1.39 Gm./100 ml. (geometric mean), and the plasma protein concentration in these animals averaged 7.21 Gm./100 ml. (Table II). The red cell count averaged 6,300 cells/mm.^{3} and the white cells 2,820 cells/mm.^{3} The differential

count showed 28 per cent neutrophils, 56 per cent lymphocytes, and, 16 per cent other cells, compared with 70 per cent neutrophils, 16 per cent lymphocytes, and 14 per cent other cells in the venous blood.

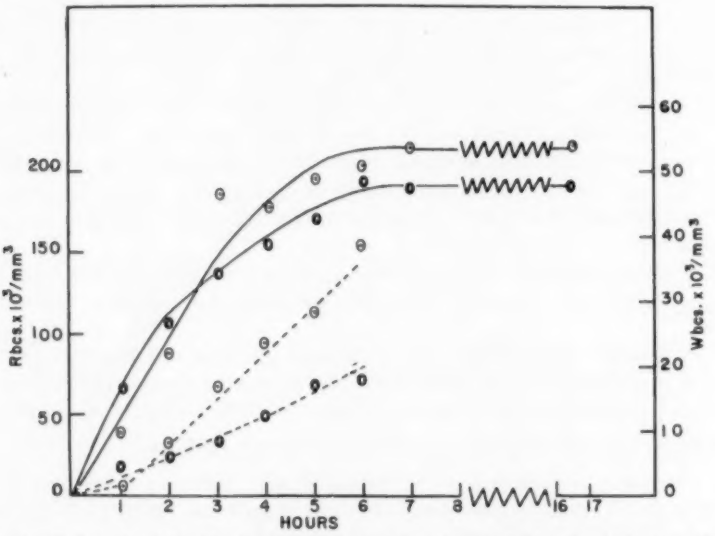

Fig. 9.—Red and white blood cell counts in the pleural fluid after injection of fresh heparinized plasma or stored serum into the pleural cavity. Mean values of 8 experiments. Key: ⊙——⊙ = white blood cells after stored serum; O——O = red blood cells after stored serum; ⊙- - - -⊙ = white blood cells after fresh heparinized plasma; O- - - -O = red blood cells after fresh heparinized plasma; left ordinate = red blood cell count/mm.³; right ordinate = white blood cell count/mm.³; abscissas = time in hours.

b. After the injection of fresh plasma: The protein concentration fell slowly, from an average of 6.7 Gm./100 ml. (8 determinations) in the plasma injected intrapleurally, to 5.9 Gm./100 ml. after 9 hours. The cell count in the fluid rose steadily (Fig. 9); in 6 hours the red cells reached a concentration of 72,000 cells/mm.³, the white cell concentration was 39,000 cells/mm.³ and both were still rising. The differential white count showed a greater proportion of neutrophils (79 per cent) and fewer lymphocytes (10 per cent) than in naturally-occurring pleural fluid, and also contained 3 per cent of basophil cells.

c. After the injection of stored serum: When stored serum was introduced into the pleural cavity, the protein concentrations had attained a constant value 79 ± 6 per cent of the circulating plasma level after 7 hours and showed no further change in the ensuing 8 to 12 hours. The cell counts in the pleural fluid rose rapidly after the injection of stored serum (Fig. 9) and reached stable levels after 6 hours that were unaltered after a further 10 hours. The red cell count reached 192,000 cells/mm.³, and the white count was 54,000 cells/mm.³ The rate of cell entry into the pleural cavity after the intrapleural injection of stored serum was several times faster than when fresh heparinized plasma had been so injected.

The average differential count was similar to that found after the injection of fresh plasma, averaging 80 per cent neutrophils, 12 per cent lymphocytes, 4 per cent monocytes, and 4 per cent basophils. The relatively high percentage of basophils is noteworthy. Many of these cells were typical "mast cells," being 2 to 3 times the size of the neutrophil with a large pale nucleus and with cytoplasmic granules giving a metachromatic reaction with toluidine blue. Fig. 10 shows an experiment in which fresh heparinized plasma was initially injected into the pleural cavity. After 8 hours when the cell counts were becoming stabilized 20 ml. of stored serum was injected. There was an immediate increase in the rate of cell entry and the white cell count reached 82,000 cells/mm.^{3} after 1 hour.

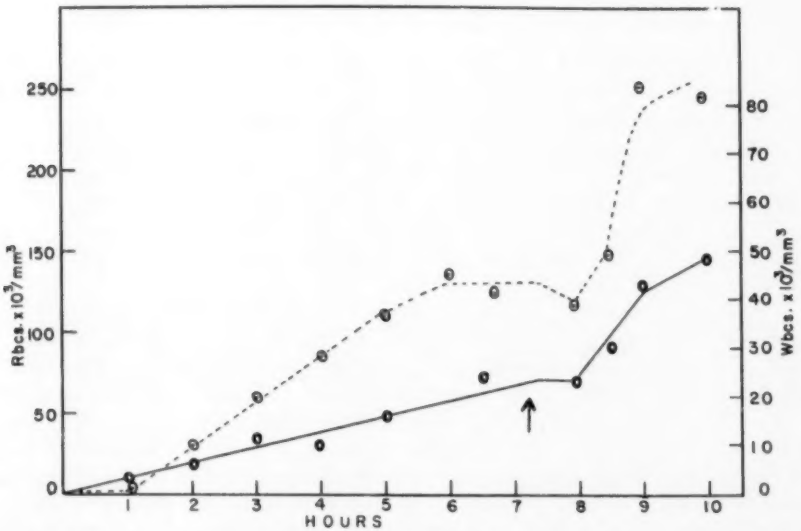

Fig. 10.—Same experiment as in Fig. 6. Effect of stored serum on cell entry. At the arrow, 20 ml. of stored serum was injected into the pleural cavity.
Key: O——O = red cell count; ⊙- - - -⊙ = white cell count; left ordinate = red cell count/mm.^{3}; right ordinate = white cell count/mm.^{3}; abscissas = time in hours.

d. After the injection of isotonic saline: The protein entry into the pleural cavity after the injection of isotonic saline has been discussed previously. The magnitudes of the cell counts were directly related to the rate of entry of protein. In the majority of dogs with a low rate of protein entry the red cells averaged 17,250 cells/mm.^{3} and the white cells 15,000 cells/mm.^{3} after 6 hours. In the 3 dogs that showed a high rate of protein entry, the red cells reached 150,000 cells/mm.^{3} and the white cells 94,800 cells/mm.^{3} at 6 hours.

e. Rate of removal of reticulocytes: In two experiments a red cell suspension in which a high proportion of the cells were reticulocytes was injected into the pleural cavity. The reticulocyte-rich cells were obtained by treating another dog with acetylphenylhydrazine for 5 days. After making allowance for maturation of the reticulocytes, the total reticulocytes had decreased from 1.97×10^9 to

0.70×10^9 in 6 hours in one experiment and from 2.0×10^9 to 0.60×10^9 in the other. Owing to the inaccuracies of the method it was not possible to obtain satisfactory quantitative measurement of cell removal, but the rate was of the same order as that of the suspending fluid. These experiments gave no indication there was any greater hindrance to the entry of red cells into the lymphatics than there was for the pleural fluid.

DISCUSSION

The lymphatic drainage of the pleural cavity behaves in a simple manner. The lymphatic flow at rest averaged 0.57 ± 0.14 (S.D.) ml./Kg. hr. and was unaffected by the composition of the pleural fluid or by wide variations in the amount of fluid in the pleural cavity. This lymphatic flow corresponds to a turnover through the pleural cavity of 27 per cent of the dog's plasma volume per day.

The lymphatic flow was changed significantly only by alteration in the rate and depth of respiratory movements. Thus hyperventilation produced by inhalation of carbon dioxide, increased the lymphatic flow up to fourfold, and hypoventilation associated with anesthesia, depressed the lymphatic flow. These results agree with the observations of Courtice and Simmonds[5] on the lymphatic flow in the cannulated thoracic and right lymphatic ducts of the cat. It is probable that lymph movement in the pleural lymphatics, as in lymph channels in other parts of the body, is passive and depends on the movement of the surrounding structures.

Following a period of hyperventilation it was noted that the lymphatic flow remained consistently raised above the control level despite a rapid return of respiratory movements to normal. We have no evidence of the maximum duration of this effect, but this elevated lymphatic flow has been observed to continue for periods up to 8 hours without showing signs of returning to the control level. The simplest explanation of the phenomenon is that in the resting animal a proportion of the lymphatic channels leading from the pleural cavity were not patent. During hyperventilation, the increased respiratory excursions caused these previously inactive vessels to become patent and these vessels remained open after the period of hyperventilation was over and accounted for the persistent high lymphatic flow.

This observation may be of some importance in the management of human effusions, in that rest in bed may delay absorption, whereas quite short periods of activity might have a disproportionately large effect due to the persistence of a raised rate of absorption.

The interchange of fluid between the blood and pleural fluid is complex. It is dependent on the hydrostatic pressure gradient across the tissues separating the two fluids to which gradient the intrapleural pressure contributes, and also on the composition of the pleural fluid, and the presence in it of substances modifying capillary permeability.

When fresh heparinized plasma was placed in the pleural cavity, protein entered the fluid from the blood, as shown by the steady decrease in the specific activity of the intrapleural protein label (i.e., in the amount of T1824 or

I^{131}-labeled bovine albumin per gram of pleural fluid protein). From this change in specific activity of the protein in the pleural cavity the rate of entry of new protein was calculated, and found to average about 20 mg./Kg. hr. Since the rate of fluid entry in these experiments was 0.47 ml./Kg. hr., this meant that the concentration of the protein in this entering fluid was 4.3 Gm./100 ml. The protein concentration in the circulating plasma averaged 7.1 Gm./100 ml. and therefore the concentration of protein in the fluid entering the pleural cavity was 62 per cent of that in the blood plasma.

This reduced protein concentration could be due to molecular filtration in isoporous capillaries or due to filtration in heteroporous capillaries in which two ranges of pore size coexist. In the second case, which we consider the more likely, the smaller pore can be regarded as completely impermeable to protein whereas the larger pore offers no hindrance to the passage of protein and permits the filtration of fluid with the same protein concentration as in the blood plasma. The pleural fluid would result from the mixture of these two filtrates. The first explanation is unacceptable because it would involve a greater holdback of the large radius protein molecules than of the small radius protein molecules and thus should lead to fluid rich in albumin and relatively poor in gamma globulin as compared with the blood plasma. Luetscher[13] has shown that this is not the case in either pleural or peritoneal effusions in man. We have found that the protein entering saline placed in the pleural cavity was very similar in electrophoretic pattern to that in the blood plasma. Further, if the pores in the pleural capillaries were of a critical diameter for protein molecules, they would not readily admit such large bodies as red and white cells, yet we have found relatively high concentrations of both these cells entering the pleural fluid. These considerations lead us to believe that fluid enters the pleural cavity through two different sized pores in the pleural capillaries.

As a result of a study of the movement of dextran molecules of different sizes across the blood-lymph barrier, Grotte[14] had found evidence that capillaries in the dog's leg, cervical region, and liver contain pores of two different sizes. The proportion of dextran molecules entering the lymph was independent of molecular weight in the range of 35,000 to 360,000. With molecules smaller than 35,000 the lymph/plasma concentration ratio increased proportionately to the reduction in molecular weight. Grotte concluded that there were two different pore sizes in the capillaries, the smaller of which did not admit dextran molecules greater than 35,000 molecular weight and the larger which was not selective even for the highest molecular weight (360,000) which was tested. The large pores, however, held back microspheres of acrylic plastic with a radius of 360 Å.

Since it would appear that the entering fluid had about 62 per cent of the protein concentration found in the blood plasma, the two-pore hypothesis requires that 62 per cent of the fluid came through the large pores, and the remaining 38 per cent of fluid was a protein-free filtrate which came through the small pores. Fluid formation through the large pores will be a bulk flow

and not dependent on colloid osmotic pressure, since a colloid osmotic pressure cannot be established across a membrane whose pores are freely permeable to protein. The fluid movement through these large pores will, therefore, depend on (1) the net hydrostatic pressure normal to the pores; (2) the number of the large pores; and (3) the fourth power of the mean pore diameter, since this is a hydrodynamic flow.

Fluid movement through the small pores, which are impermeable to protein, will be influenced also by the colloid osmotic pressure gradient from capillary blood to pleural fluid in addition to the factors listed above. The colloid osmotic pressure in the extracellular space surrounding the pleural capillaries lies presumably between that of the newly formed fluid and that of the pre-existing pleural fluid. When the fluid in the pleural cavity is blood plasma there will be only a trivial difference of colloid osmotic pressure across the capillary wall; therefore, colloid osmotic pressure may be ignored in considering the factors controlling fluid movement. It was found that when the intrapleural pressure exceeded 2 mm. Hg, fluid movement into the pleural cavity ceased. This suggests that the pressure in the pleural capillaries is about 2 mm. Hg. This is the order of pressure found in the capillaries in the pulmonary circuit, and it may be that the pleural capillaries in the dog are supplied from the pulmonary circulation. Miller[15] came to the same conclusion from anatomical studies, but pointed out that the bronchial circulation played a major role in supplying vessels in the visceral subpleural region in man. The parietal pleural capillaries are systemic and presumably the mean intracapillary pressure is of the order of 30 mm. Hg and a rise in intrapleural of +2 mm. Hg would still leave a considerable hydrostatic pressure gradient across the capillary wall. The complete cessation of entry of fluid into the pleural cavity when the intrapleural pressure was so raised can only mean that the parietal pleural capillaries play a negligible role in the movement of fluid into the pleural cavity.

The mean intrapleural pressure in the resting dog was –5 to –8 mm. Hg so that the net hydrostatic pressure available for formation of fluid was about 7 to 10 mm. Hg. This net pressure is presumably the over-all driving force for movement through the large pores, and for movement through the small pores this force is reduced by whatever colloid osmotic pressure difference is available. It seems reasonable to assume that with a raised intrapleural pressure the pleural fluid formation will cease when the intrapleural pressure negates the hydrostatic force driving the fluid through the pores. On the other hand, increase in the pressure gradient from capillary blood to pleural fluid produced by reduction of the intrapleural pressure or increase in the pulmonary capillary pressure will lead to an increase in pleural fluid formation. These findings indicate the importance of the normally negative intrapleural pressure in the formation of pleural fluid. Thus as the fluid accumulates in a pleural effusion, the rise in the intrapleural pressure will retard the further formation of fluid. Conversely a more negative pressure associated with atelectasis will predispose to pleural fluid accumulation.

If we now turn to the effect of pleural fluid colloid osmotic pressure, this is well illustrated by the experiments with isotonic saline. In the more common type of response in dogs to the intrapleural injection of isotonic saline, fluid entry was reduced to a very low level or abolished. In two cases the direction of flow was reversed so that the net movement of fluid was in the direction of pleural fluid to blood. However, the rate of protein entry was very similar to that found when plasma was used. This is in agreement with the hypothesis that the rate of movement of fluid through the large pores is virtually independent of the nature of the fluid. The reversal of the flow can be attributed to the lowering of the colloid osmotic pressure in the pleural fluid as the result of the dilution with saline. The difference in colloid osmotic pressure between capillary blood and pleural fluid becomes such that the hydrostatic pressure responsible for flow into the pleural space is more than balanced and flow occurs from the pleural fluid to capillary. The over-all picture is therefore that movement of fluid through the large pores, which is unaffected by colloid osmotic pressure changes, is approximately balanced by reabsorption of fluid from the pleural cavity through the small pores.

In the 3 dogs that responded to the intrapleural injection of isotonic saline by a large movement of fluid into the pleural cavity, there was a great increase in the rate of protein entry, 93 mg./Kg. hr. as compared to 20 mg./Kg. hr. in the other dogs. This must mean that an increase in capillary permeability occurred and this suggestion is supported by the increase in the entry of red and white cells as well as of protein. The explanation of this change in permeability is not clear at present.

Apart from hydrostatic and colloid osmotic pressures, a major factor in fluid movement is the permeability of the capillary wall. This is greatly increased by histamine and also by a substance present in stored serum. The response to stored serum was unaffected by antihistamines in doses that abolished the response to histamine and histamine liberators. Miles and Wilhelm[16] found that storage or dilution of guinea pig serum led to the development of substances increasing the permeability of skin capillaries after intradermal injection. We have had similar results with dog plasma. These substances may account for the increased permeability of the pleural capillaries found after the intrapleural injection of stored serum. If such substances are generated in vivo they may be of the greatest importance in initiating and perpetuating fluid accumulation in serous cavities.

The transfer of low molecular weight water soluble substances between blood and pleural fluid is not restricted to the rate of net fluid transfer. The movement seems to be determined by simple diffusion considerations, and like such movements elsewhere in the capillaries it may be either "diffusion limited" for the moderate sized molecules, or "blood flow limited" for the smallest molecules. Our evidence suggests that equilibration of water or Na^{22} is mainly limited by pleural blood flow, and that the clearance rate of Na^{22} introduced into the pleural cavity gives an approximate measure of the total pleural blood

flow. This was of the order of 70 to 80 ml./Kg. hr. This very large clearance rate for substances of molecular weights of a few hundred contrasts sharply with the rates for substances of large molecular weight of the order of the plasma proteins. These large molecules are removed solely by the pleural lymphatics and hence the time course for their absorption is prolonged.

We have obtained some information on the factors controlling the turnover of cells. The experiments using naturally-labeled red cells (reticuloeytes) show that the lymphatic vessels draining the pleural cavity offer no greater hindrance to the removal of red cells than they do to plasma proteins. No specific information was obtained as to the existence of a restriction to white cell removal.

The rate of entry of cells into the pleural fluid was strongly influenced by the nature of the fluid introduced. After the injection of fresh heparinized plasma, the red cells entered the pleural cavity at an average rate of 1.5×10^8 cells/Kg. hr., and the white cells entered at a rate of 0.7×10^8 cells/Kg. hr. The cell counts were still rising at the end of 6 hours at which time the white cell count was of the order of 40,000 cells/mm.^{3}, about 4 to 5 times the white cell count of peripheral blood. When stored serum was injected both types of cells entered at a faster rate during the first 3 to 4 hours than after fresh heparinized plasma. In the case of red blood cells, the rate was 12×10^8 cells/Kg. hr., some 8 times faster than after fresh heparinized plasma, and the white cell entry was 2.8×10^8 cells/Kg. hr. which was about 4 times faster than after heparinized plasma. Following this rapid entry a steady state was reached at about 54,000 white cells/mm.^{3} and at 192,000 red cells/mm.^{3} Thus the red cell count in the pleural fluid was only 4 per cent of the peripheral blood count, whereas the concentration of white blood cells was about 6 times that found in the peripheral blood. While it would be quite reasonable to explain the steady concentration of red cells reached in the pleural fluid as the attainment of a steady state in which a passive movement of red cells from blood to pleural fluid balances the rate of red cell removal by the lymphatics, no such simple explanation can be offered for the behavior of the white blood cells. It is more likely that the polymorphonuclear leukocytes and the lymphocytes are being attracted into the pleural fluid by the generation of a chemotactic substance. It is surely surprising that when the ratio between red cells and white cells in the peripheral blood is about 600:1, the rate of red cell entry should be only 2 to 5 times the white cell entry.

The differential white cell count in the pleural fluid was similar whether stored serum, fresh heparinized plasma, or isotonic saline had been injected. The differential count differed in two respects from that found in the peripheral blood. The proportion of neutrophils was somewhat higher, and the percentage of basophils was much higher, occasionally reaching 10 per cent of the total count in the pleural fluid. Many of these basophils were large and immature in appearance, resembling more closely tissue mast cells than the basophils seen in peripheral blood. Presumably the basophils in the pleural fluid are derived from the collections of such cells that have been noted in the pleura by a number of workers.[17] Padawer and Gordon[18] have noted the

presence of numerous mast cells in the peritoneal fluid of the rat, and have used this fluid for the preparation of pure mast cell suspensions. Courtice, Harding, and Steinbeck[19] have also commented on the presence of mast cells in the peritoneal fluid of the rat.

The present methods of measuring fluid turnover necessitate the introduction of fluid into the pleural cavity and there is no certainty that these results can be applied quantitatively to the normal pleural cavity which contains only a trace of fluid. Indeed the low-protein concentration and low-cell counts found in normal fluid argue against this. Similar low values for protein and cell concentrations were found in naturally occurring pleural fluid obtained from healthy men by Yamada.[20] Maurer, Warren, and Drinker[21] also found that the protein concentration of naturally occurring fluid in the peritoneal and pericardial cavities was also low. The high concentration of cells and the high rate of protein entry found in all our experiments suggest that the permeability of the pleural capillaries was increased; since this increased capillary permeability occurred whatever the composition of the fluid introduced into the pleural cavity, it is probably due in part to the simple expansion of the pleural fluid volume. The mechanism mediating this is quite obscure at the present time, and, while the present results may have a direct bearing where fluid has accumulated in the pleural cavity, they will need modification when the normal small amounts of fluid are present.

SUMMARY

1. When fresh heparinized blood plasma was injected into the pleural cavity of the dog, the lymphatic flow at rest was 0.57 ± 0.14 (S.D.) ml./Kg. hr. which was nearly balanced by fluid entering the pleural cavity from the capillaries at a rate of 0.47 ± 0.10 (S.D.) ml./Kg. hr.

2. The lymphatic flow was increased by hyperventilation and decreased by hypoventilation. Following hyperventilation, the lymphatic flow was elevated for many hours.

3. The fluid entering the pleural cavity was rich in protein.

4. The entry of fluid was greatly increased by histamine and substances present in stored serum which increased capillary permeability.

5. In most animals intrapleural injection of isotonic saline decreased or reversed the transfer of fluid from blood to pleural fluid. In a minority of animals a great increase in fluid entry occurred.

6. An increase in intrapleural pressure to 2 mm. Hg or more above atmospheric pressure abolished fluid entry. Reduction of intrapleural pressure increased fluid entry.

7. Cell counts in the pleural fluid after the injection of fluid became high tending to 30,000 to 60,000 white blood cells/mm.^{3} and 100,000 to 200,000 red blood cells/mm.^{3}

8. Evidence is presented that the pleural capillaries from which fluid passes to the pleural cavity, may be derived from the pulmonary circulation

and may contain two types of pore; the larger pore allowing free passage of plasma protein and restricting the passage of cells and the smaller pore being impermeable to protein.

9. Low molecular weight substances pass rapidly in either direction between capillary blood and pleural fluid. The clearance of Na^{22} and water introduced into the pleural cavity gave a lower limit of 80 ml./Kg. hr. for the pleural blood flow.

10. Normal pleural fluid has been collected. The average volume for the dog was 1.3 ml. The average protein concentration was 1.39 Gm./100 ml.; the red cell count 6,300/mm.^{3} and the white cell count 2,820/mm.^{3}

We would like to thank Dr. Hugh E. Burke for his advice.

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NOTE ON SILENT SARCOIDOSIS OF THE GASTRIC MUCOSA

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SARCOIDOSIS of the stomach, believed to be unusual, manifests itself mainly through granulomatous and fibrotic obstruction of the pyloric outlet.[1, 4, 5, 14, 16, 18] Hemorrhage from stomachs with submacroscopic mucosal sarcoidosis is well known although rare.[17] In several reported cases, gastric involvement was encountered only by chance at autopsy or upon gastric resection for presumably unrelated ulcer or cancer.[2, 3, 6-13] Actually, the incidence of gastric localization is uncertain, both because of the nonspecific histopathologic nature of the granuloma and because miliary mucosal involvement without gross changes is apparently the commonest form for gastric sarcoidosis to assume.

Because of the diagnosis of one case of sarcoidosis through transoral gastric mucosal biopsy,[17] biopsy specimens taken in a series of patients being investigated for sarcoidosis were studied for incidence of mucosal sarcoidosis and for histopathologic effects of silent sarcoid granulomas on the gastric mucosa.

MATERIAL AND METHOD

Single gastric mucosal biopsy specimens were taken by vacuum tube[15] on 74 occasions from 60 patients who were eventually diagnosed as sarcoidosis by the usual clinical and histopathologic criteria. Superficial lymph nodes, scalene fat pads, and liver biopsies had furnished the material for pathologic examination. All of the patients were men, between 18 and 33 years of age. There were 44 Negroes and 16 Caucasians. At the time of gastric mucosal biopsy, all of the patients were examined gastroscopically and 33 of the 60, esophagoscopically. None of the patients had symptoms which suggested upper gastrointestinal disease, and, in fact, the majority did not consider themselves sick, having come under medical investigation only because of abnormalities discovered on routine chest roentgenography.

The biopsy specimens were taken close to the greater curvature, about in line with the esophageal axis. They represented, therefore, mucosa of the fundic type. The specimens before fixation were uniform disks measuring approximately 5 mm. in diameter and containing full mucosal thickness plus a little of the submucosa. The tissue was processed in the routine fashion in the laboratory; each specimen was bisected before embedding and 4 sections were studied, unless their contents called for examination of more.

RESULTS

The preliminary upper gastrointestinal x-ray studies failed to reveal gastric disease in any of the 60 patients. No esophagoscopic abnormalities were found

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other than occasional infringement on the esophageal lumen by enlarged mediastinal lymph nodes.

Gastroscopically, abnormality was found in only 6 patients. Two had the typical changes of generalized atrophic gastritis, and in 4 there were the findings often labeled "superficial gastritis": irregular areas of hyperemia, an excess of mucus and thin gray exudate, and a few erosions. Repeated gastroscopic examinations were made in these 6 cases over periods up to 18 months, without changes in the 2 patients with atrophic gastritis but with fluctuations toward and away from normal in the others.

Sarcoidosis was found in the biopsy specimens from 6 of the 60 patients. These were not the 6 patients who showed gastroscopic abnormalities. Altogether 16 specimens were taken from the 6 positive patients over periods up to 18 months, and 14 of these contained sarcoid granulomas. The passage of time brought no important change in histopathologic appearances.

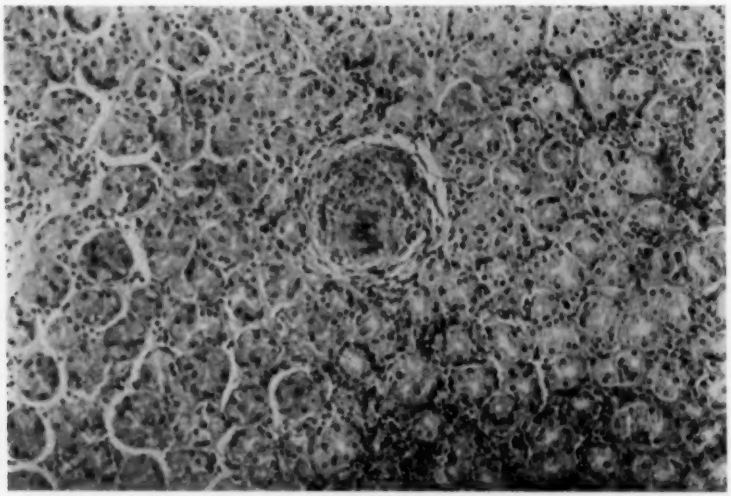

Fig. 1.—Tiny sarcoid granuloma in the glandular portion of the mucosa. Notable are the absence of peripheral reaction and the normalcy of the neighboring gastric glands. (Magnification ×100; reduced ⅓.)

The granulomas found in the gastric mucosa were entirely similar in appearance to those observed in other tissues, although all of the individual lesions were small as well as scattered. Sometimes there were 6 or 8 tiny granulomas in one section, quite separate from one another. Most were located about midway between the muscularis mucosae and surface. Fewer lay in contact with the muscularis mucosae. In the former location there was never any degree of inflammatory reaction around the granulomas or any disturbance in the neighboring gastric glands (Figs. 1 and 2). When granulomas rested against the muscularis mucosae, there often was round-cell infiltration in the area (Fig. 3).

No submucosal granulomas were found. It appeared that the sarcoidosis was disturbing the stomach very little and that there was nothing progressive about the gastric disease in these patients.

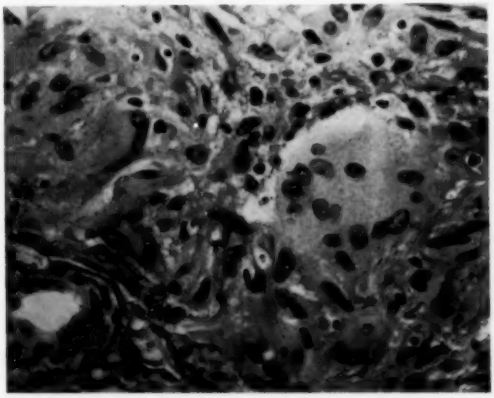

Fig. 2.—Details of another granuloma. (Magnification ×430; reduced ⅓.)

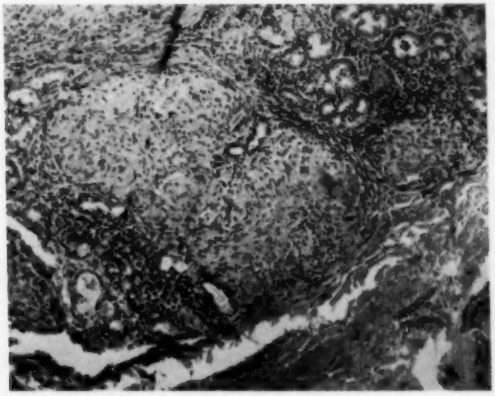

Fig. 3.—Granuloma lying on the muscularis mucosae. In this position there is a degree of peripheral round-cell infiltration. (Magnification ×100; reduced ⅓.)

COMMENT AND CONCLUSION

As was pointed out at the start, sarcoidosis of the stomach can produce serious illness. The present experience suggests that silent involvement of the gastric mucosa may not be excessively rare, even when the matter is judged by a sampling technique which depends on diffuse involvement for a positive result. The implication to be drawn from the observed incidence, the appearance of the lesions, and the failure of histopathologic progression in serial specimens is that mucosal sarcoidosis does not often advance to the point at which serious clinical consequences, such as pyloric obstruction and mucosal hemorrhage, might occur.

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PROTEIN REQUIREMENTS IN CHRONIC RENAL INSUFFICIENT PATIENTS

A Study of the Nitrogen Minimum

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VALUES for the minimum nitrogen requirement for human beings have been carefully investigated in normal individuals.[1-5] The procurement of such data has been facilitated by the observation that the relation between nitrogen balance and absorbed nitrogen when the individual is in negative balance is linear.[6] The extrapolation of balance data back to the level of nitrogen equilibrium will give the minimum nitrogen requirement for equilibrium.

These values are related to the amino acid pattern of the dietary source and range in excess of 2 mg. absorbed nitrogen per basal calorie per 24 hours.[7] A protein source of unknown deficiency, such as whole egg protein, results in a lower requirement than a source deficient in some known amino acid. In the case of the deficient source, the requirement may be lowered by supplementation with the limiting factor as in the experiment reported by Bricker and associates[6] in which, on a white flour diet, an adult college female was in marked negative nitrogen balance. When the diet was supplemented with lysine, the urine nitrogen dropped quickly by an amount 350 per cent greater than the added lysine and the nitrogen balance became positive. Deficiency is a relative rather than an absolute term. A protein may be deficient for growth, but adequate for maintenance. The requirements may be different in health and disease. The relationship between endogenous nitrogen loss and basal heat production may be altered by differences in age, sex, and prior treatment.[8]

The patient with advanced impairment in renal function has a reduced ability to excrete end products of protein metabolism. To compensate for reduced renal function, the plasma concentration of nitrogen and other nonvolatile catabolites rises. Whether or not the accumulation of nitrogen adversely affects the health of the organism is a moot question. Most sources of protein contain, in addition to amino nitrogen, sulfate and phosphate radicals, purines, and other extractives, all of which require renal excretion. Ideally, it would seem that a minimum of renal function would be required

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when the diet contained the required amino acids in the proportions necessary to provide optimal efficiency of utilization. A protein deficient diet with adequate or excess calories from carbohydrate and fat will affect a maximal reduction in the metabolism of nitrogen and thus reduce the protein catabolites requiring excretion. This regimen has been employed as a temporary measure in the treatment of acute renal insufficiency and is advocated by some for the long-term care of the chronically renal insufficient.[8] Prolonged protein depletion is not without consequence and will eventually produce illness of itself. The chronic renal insufficient patient may survive for many years and treatment should be so regulated as to maintain the patient indefinitely. Ideally, efforts to minimize protein catabolism would not include protein restriction to the point of producing a negative nitrogen balance.

Some information is available on the nitrogen requirements of human beings in abnormal states. The requirement is much higher in the sequence of events immediately following severe trauma, burns, and the crush syndrome.[9-11] Pregnancy, hyperthyroidism, and fever all increase the minimum protein requirement as may be inferred by their influence on basal oxygen consumption. There have been conflicting reports on the nitrogen requirement in the aged[12-14] and in the presence of renal insufficiency.[8, 15-17] These latter observations together with the opinions of many observers on the phenomena of "toxic destruction of protein" in azotemia due to renal insufficiency stimulated the following investigations of the nitrogen minimum in chronic renal insufficient human beings.

METHODS

The renal insufficient patients selected for use in this study were chosen after preliminary periods of observation varying from 1 to 3 weeks. These patients were apparently free of infection, gross bleeding, or purpura at the time of study. They were maintained in rooms adjoining the special diet kitchen. No more than two patients were studied at any one time. Pertinent clinical observations concerning the patients are summarized in Table I.

Standard nitrogen balance procedures were followed using the recommendations of Bauer and Aub.[18] The preliminary evaluations included the clinical history, course, duration and manifestations of the disease process leading to renal insufficiency, a physical examination, and a laboratory evaluation. The latter included a complete blood count, plasma total proteins, and albumin-globulin ratio, nonprotein nitrogen, creatinine, total calcium, inorganic phosphorus, electrolytes, protein bound iodine, I^{131} uptake, basal oxygen consumption, chest x-ray, and a 12-lead electrocardiogram. Blood pressure, pulse, and temperature were measured twice daily. Other studies were made in certain instances. The patients were weighed daily at 8:00 A.M. after voiding and before breakfast. Activity was unrestricted, but, once a pattern was established, it was not varied. All patients spent from 14 to 16 hours daily in bed, the remainder about equally divided between ambulation and sitting.

Caloric requirements were estimated at from 27 to 30 calories per kilogram of body weight per day. The carbohydrate to fat ratio in the diet was likewise estimated and was maintained at a 3 to 1 ratio. The diets were constant in regard to minerals and vitamins*

*Diets were supplemented with vitamins: vitamin A, 5,000 units; vitamin D, 500 units; vitamin C, 150 mg.; thiamin, 10 mg.; riboflavin, 10 mg.; pyrodixin, 2 mg.; calcium pantothenate, 20 mg.; nicotinamide, 100 mg.; folic acid, 1.5 mg.; vitamin B_{12}, 1.5 mEq.; cobalt, 0.1 mg.; copper, 1.0 mg.; iron, 10 mg.; manganese, 6 mg.; magnesium, 1.0 mg.; molybdenum, 0.2 mg.; zinc, 1.5 mg.; calcium, 60 mg. (Unicap, therapeutic: The Upjohn Company, Kalamazoo, Mich.)

insofar as possible. The low-protein diets were nearly always low in calcium and were supplemented with calcium lactate to provide at least 600 mg. calcium daily. Egg albumin diets were low in phosphorus, but no effort was made to regulate this moiety. The sodium was constant for each individual, but varied from 900 to 2,200 mg. per day in different patients. Potassium intake varied moderately at different dietary levels of protein.

TABLE I. SUMMARY OF CLINICAL AND LABORATORY DATA FOR FIVE CHRONIC RENAL INSUFFICIENT PATIENTS AND ONE HEALTHY SUBJECT

	SUBJECT	1	2	3	4	5	HEALTHY SUBJECT
	Age	38	46	56	54	20	32
	Sex	M.	M.	F.	M.	M.	M.
	Height (cm.)	177	169	159	167	179	176
	Weight (Kg.)	82.1	66.8	58.6	92.2	63.2	71.8
	Blood pressure	220/138	222/142	150/80	180/110	194/118	110/70
	Basal cal./M^2/hr.*	39.2	30 3	31.0	33.9	37.3	34.6
PLASMA	NPN (mg.%)	84.0	86.5	45.5	54.5	103.5	27.0
PLASMA	Creatinine (mg.%)	11.0	7.66	2.20	3.85	11.3	1.34
PLASMA	Total protein (Gm.%)	7.1	5.84	6.7	7.6	4.33	6.67
PLASMA	Albumin (Gm.%)	4.8	3.64	4.1	4.9	2.77	4.53
PLASMA	Calcium (mg.%)	10.25	9.55	10.02	10.22	7.70	10.89
PLASMA	Inorganic phosphorus (mg.%)	7.25	7.32	4.40	4.53	11.33	4.10
PLASMA	CO_2 (mEq./L.)	17.7	18.1	22.0	25.3	17.1	27.9
PLASMA	Chloride (mEq./L.)	108.5	102.0	105.9	101.4	111.0	102.0
PLASMA	Sodium (mEq./L.)	140	139	138	138.6	142.5	145
PLASMA	Potassium (mEq./L.)	4.4	4.7	4.2	4.9	4.7	4.5
VENOUS BLOOD	Hemoglobin (Gm.%)	12.1	9.8	13.2	11.5	7.5	15.4
VENOUS BLOOD	Hematocrit (%)	36	29	40	35	22	49
VENOUS BLOOD	R.B.C. (millions c.mm.^{3})	3.93	4.09	4.55	3.95	2.46	4.90
VENOUS BLOOD	W.B.C. (c.mm.^{3})	6,700	7,400	7,500	7,300	6,850	9,850
	Clinical diagnosis	Chronic glomerulonephritis		Polycystic disease	Chronic glomerulonephritis		Healthy subject

*Based on oxygen consumption and according to Du Bois' standards.

Investigation of all patients was begun on a diet of 1.0 Gm. protein per kilogram of body weight per day after equilibration periods varying from 4 to 6 days. Following equilibration periods, collections were begun using collection periods of 3 or 4 days.

Urine was collected in 24-hour periods with chloroform as a preservative. The urine was refrigerated (4° C.) at all times. On 24-hour specimens total volume, specific gravity, and creatinine were determined.[19] Provided the creatinine values were constant, the 24-hour samples were pooled to provide aliquots of the urine. These in turn were analyzed for total nitrogen by the method of Kjeldahl.[19] Proteinuric nitrogen was determined by Kjeldahl analysis of a trichloroacetic acid precipitate of urine prepared according to a minor modification of the method of Hiller and co-workers.[20] Stool markers were either carmine, 0.5 Gm., or charcoal. Stools were collected directly into numbered and weighed evaporating dishes, 12 inches in diameter, covered, and immediately refrigerated. The collection was weighed and then transferred to a sealed plastic container; the stool weight was determined by the difference from the weight of the collection dish. When the 3- or 4-day sample was accumulated, it was homogenized and a representative aliquot analyzed for total nitrogen by the method of Kjeldahl.

The nitrogen ingested as coffee or tea was calculated from their weight. Tea and coffee were made each day in the same fashion; therefore, after several analyses, values were calculated using 249 mg. nitrogen per 1,000 Gm. coffee and 97 mg. nitrogen per 1,000 Gm. tea. These sources are not homogenous and so the average is more representative

TABLE II. NITROGEN BALANCE DATA FOR FIVE RENAL INSUFFICIENT PATIENTS AND ONE HEALTHY SUBJECT

DAYS	DIET*	INGESTED N (TOTAL SUPPL.)		TOTAL FECAL N (GM./D.)	ABSORBED N (GM./D.)	URINARY NITROGEN: PROTEIN (GM./D.)	URINARY NITROGEN: NON-PROTEIN (GM./D.)	N FOR GROWTH (GM./D.)	NONPROTEINURIC BALANCE: MG./B.C.†	NONPROTEINURIC BALANCE: GM./D.
Patient 1										
6	a									
4	a	12.32		1.57	11.81	0.15	8.99	1.10	+0.30	+0.56
3	b									
4	b	10.63		1.61	10.08	0.13	7.63	1.09	+0.16	+0.30
4	b	11.16		1.87	10.25		7.53	1.09	+0.30	+0.67
9	c									
3	c	5.45		1.61	4.90		4.92	1.10	−1.19	−2.18
3	c	5.47		2.14	4.39		4.20	1.10	−1.07	−1.97
3	c	5.52		1.67	4.91	0.14	4.53	1.10	−0.97	−1.78
3	c	5.47		1.71	4.82		4.69	1.10	−1.11	−2.03
5	a									
3	a+	5.87		1.94	4.99		4.58	1.09	−0.98	−1.79
3	a+	6.63		1.66	6.03	0.36	4.37	1.08	−0.68	−1.24
3	a									
3	a+	2.00		1.18	1.88		4.66	1.08	−2.70	−4.92
3	a+	2.06		1.54	1.58	0.31	4.17	1.08	−2.61	−4.73
3	aA									
3	aA	5.69	0.41	1.13	5.62		5.66	1.07	−1.22	−2.17
3	aA	5.83	0.41	1.76	5.13	0.25	6.31	1.07	−1.86	−3.30
3	aC									
3	aC	6.01	0.43	1.51	5.56		5.31	1.06	−1.06	−1.81
3	aC	6.14	0.43	1.52	5.68	0.20	5.28	1.06	−0.97	−1.71
Patient 2										
6	b									
3	b	9.17		1.56	8.30	0.47	6.94	0.96	−0.23	−0.29
13	a									
3	a+	5.84		1.47	5.06		3.85	0.95	−0.72	−0.88
3	a+	5.65		1.04	5.30	0.23	3.75	0.95	−0.27	−0.33
3	a+	5.52		1.22	4.99		4.59	0.95	−1.01	−1.24
3	aE	6.41	0.70	1.02	6.08		5.90	0.95	−1.19	−1.46
3	aE	6.21	0.70	0.96	5.94	0.36	5.70	0.95	−1.70	−1.30
3	a+	1.65‡		0.74	1.60	0.26	3.76	0.95	−3.11	−3.80
Patient 3										
5	a									
3	a	9.65		0.97	9.28		8.75	0.90	−0.81	−0.97
3	a	9.68		0.89	9.40	0.06	8.19	0.90	−0.25	−0.30
8	a									
3	a	5.26		0.76	5.10		3.98	0.90	−0.30	−0.37
3	a	5.24		0.92	4.92	0.06	4.29	0.90	−0.73	−0.87
9	d									
3	d+	4.07		0.56	4.11		4.01	0.90	−1.17	−1.46
3	d+	4.07		0.60	4.07	0.12	4.33	0.90	−1.47	−1.76
3	dA									
3	dA	4.47	0.39	0.62	4.45		4.06	0.88	−0.94	−1.09
3	dA	4.45	0.39	0.66	4.39	0.04	4.47	0.88	−1.34	−1.56
3	dC									
3	dC	4.38	0.35	0.56	4.42		3.44	0.88	−0.43	−0.50
3	dC	4.63	0.35	0.64	4.59	0.02	3.41	0.88	−0.25	−0.30
3	dB									
3	dB	4.49	0.39	0.61	4.48		3.97	0.88	−0.83	−0.97
3	dB	4.38	0.39	0.61	4.37	0.06	4.17	0.87	−1.10	−1.27
3	d									
3	d+	1.83		0.67	1.76		3.52	0.86	−2.81	−3.22
3	d+	2.05		0.73	1.92	0.04	3.22	0.86	−2.44	−2.78
3	dF	1.94		0.70	1.84	0.02	3.11	0.85	−2.41	−2.72

TABLE II—CONT'D

DAYS	DIET*	INGESTED N (TOTAL SUPPL.)		TOTAL FECAL N (GM./D.)	ABSORBED N (GM./D.)	URINARY NITROGEN PROTEIN (GM./D.)	URINARY NITROGEN NON-PROTEIN (GM./D.)	N FOR GROWTH (GM./D.)	NONPROTEINURIC BALANCE MG./B.C.†	NONPROTEINURIC BALANCE GM./D.
Patient 4										
4	a									
3	a	15.23		1.48	14.71		11.22	1.14	+0.84	+1.39
3	a	15.34		1.51	14.79	0.52	12.53	1.13	+0.10	+0.17
9	a									
3	a+	6.67		1.48	6.15		5.06	1.13	−0.61	−1.00
3	a+	6.86		1.58	6.24	0.40	4.77	1.13	−0.38	−0.62
3	a									
3	a+	4.17		1.10	4.03		4.75	1.12	−1.71	−2.80
3	a+	4.43		1.20	4.19	0.34	3.95	1.12	−1.12	−1.84
Patient 5										
6	a									
3	a	11.50		0.99	11.35		6.44	0.99	+1.94	+3.08
3	a	11.41		1.23	11.02	1.81	6.71	0.99	+1.57	+2.48
12	a									
3	a+	6.80		1.56	6.08	1.69	4.68	0.98	−0.27	−0.42
3	a									
3	a+	4.97		1.65	4.16		4.41	0.99	−1.35	−2.11
3	a+	4.68		1.93	3.59	1.66	3.77	0.99	−1.28	−2.01
5	a									
3	a+	7.07		1.05	6.86		3.97	1.00	+0.66	+1.05
3	a+	7.20		1.56	6.48	1.65	4.40	1.00	+0.15	+0.24
5	aD									
3	aD	8.56	1.47	1.92	7.48		5.05	1.02	+0.35	+0.57
3	aD	8.81	1.47	1.82	7.83	1.86	5.82	1.02	+0.09	+0.15
5	a									
3	a	8.82		1.96	7.70		4.68	1.02	+0.38	+0.62
3	a	9.11		1.62	8.33	1.73	5.52	1.02	+0.58	+0.95
Healthy Subject										
6	a									
3	a	12.80		1.05	12.46		12.44	1.02	−1.13	−1.71
3	a	13.27		1.37	12.61		11.93	1.02	−0.70	−1.05
12	a									
3	a	6.96		0.77	6.90		5.40	1.01	−0.15	−0.22
3	a	7.07		0.66	7.12		6.18	1.01	−0.52	−0.78
3	a	7.29		0.88	7.12		6.26	1.01	−0.43	−0.64
10	a									
3	a+	3.83		1.15	3.37		3.08	1.01	−0.94	−1.41
3	a+	4.00		1.33	3.36		2.80	1.01	−0.77	−1.14
3	a									
3	a+	2.14		0.91	1.94		2.51	1.01	−1.54	−2.29
3	a+	2.08		1.06	1.73		2.53	1.01	−1.69	−2.52

*Lower case letters denote type of dietary protein: a = mixed diet of natural foods; b = 80 to 85 per cent of dietary nitrogen from purified egg albumin; c = 70 per cent of dietary nitrogen from purified egg albumin; d = 100 per cent of dietary nitrogen from fresh whole egg. Capital letters denote dietary supplements: A = 400 mg. glycine nitrogen per day; B = 400 mg. L-methionine nitrogen per day; C = 400 mg. nitrogen per day from a mixture composed of 75 per cent L-leucine, 13 per cent L-isoleucine, 8 per cent L-methionine, 3 per cent L-phenylalanine, and trace of L-tyrosine (Sta-mino-B provided by the Staley Company, Decatur, Ill.); D = 1,000 mg. nitrogen per day from the same mixture as "C" plus 400 mg. gelatin nitrogen; E = 153.5 mg. L-lysine monohydrochloride nitrogen, 186.5 mg. DL-phenylalanine nitrogen, 34.3 mg. DL-tryptophane nitrogen, 117.6 mg. DL-threonine nitrogen; 117.2 mg. L-leucine nitrogen, 149.5 mg. DL-isoleucine nitrogen; 191.2 mg. DL-valine nitrogen, and 235 mg. DL-methionine nitrogen per day (total: 1,134 mg. nitrogen per day); F = 500 mg. acetazolamide per day. + = diet used for preparation of the graph in Fig. 1.

†Basal oxygen consumption was measured repeatedly on these subjects and was quite constant for a given individual. Du Bois' formula was used to calculate the basal caloric requirement from the oxygen consumption.

‡Collection was preceded by a 10-day period of equilibration on this diet.

than the individual measure. A half-portion of the daily diet was prepared concomitantly and similarly to the daily diet and saved for analysis. This material was pooled for the period, weighed, homogenized, and an aliquot analyzed for total nitrogen.

The intake is given as total nitrogen per day not including coffee or tea. The absorbed nitrogen is the daily intake less the dietary stool nitrogen expressed as milligrams nitrogen per basal calorie per day. The nitrogen balance is the difference between intake and output. Output includes urine nitrogen, stool nitrogen, and a factor calculated for the "nitrogen required for growth," 0.56 Gm. nitrogen per meter square body surface area per day, according to Mitchell.[21] No adjustment or calculation was made for losses in the sweat. Environmental temperature was not precisely regulated, but overt sweating was not clinically noted. The role of sweat in nitrogen excretion in renal insufficient human beings was not assessed. Urine nitrogen was corrected for the daily coffee or tea intake by subtracting this value as nonprotein nitrogen and of no biologic significance, just as it was subtracted from the intake. An adjustment was also made for changes in the plasma nonprotein nitrogen during the collection period. Plasma was analyzed before and after each period and the following calculation was made: Total nonprotein nitrogen change per day $= \left(\begin{matrix}\text{NPN mg./L.} \\ \text{before}\end{matrix} - \begin{matrix}\text{NPN mg./L.} \\ \text{after}\end{matrix}\right) \times 70$ per cent (adjustment for total body water) $\times$ mean body weight in kilograms. If the nonprotein nitrogen rose, this calculation was added to the output; if it fell, it was subtracted. Variations here were generally of little magnitude. No other factors were considered in the calculations. The base line on each patient probably varied from time to time with changes in weight and surface area. No practical method for eliminating this, other than prolonged equilibration periods, was used in this study.

Two collection periods were usually made on each diet schedule. Dietary levels of protein included 1.0 Gm. per kilogram, 0.5 Gm. per kilogram, and 0.25 Gm. per kilogram of body weight. Between the 1.0 Gm. and 0.5 Gm. per kilogram protein diets, prolonged equilibration periods were necessary before collections were resumed in order to obtain relatively constant urine nitrogen values. When the diets were changed, even though nitrogen content was not changed, equilibration periods were never less than 3 days.

RESULTS

Nitrogen balance data for 5 patients with chronic renal insufficiency and one normal subject are presented in Table II. No subject showed significant (exceeding 10 per cent of nitrogen intake) deviation from nitrogen equilibrium on a 1.0 Gm. protein per kilogram diet. It should be noted that the 5 renal insufficient patients were persistently in negative nitrogen balance when proteinuric losses are included on an intake of 0.5 Gm. protein per kilogram of body weight over periods ranging from 21 to 40 days. This is in contrast to the healthy individual, who reached equilibrium at this dietary level after 15 days.

Endogenous urinary and fecal nitrogen were not determined directly. Calculated values for endogenous urinary and fecal nitrogen are shown in Table III. Indirect estimation of fecal nitrogen from linear retrogression curves comparing dietary to fecal nitrogen, each expressed as milligrams per basal calorie, results in the values in the third and fourth columns of Table III. The endogenous fecal nitrogen values agree closely with one another and with the observed average of 0.58 Gm. nitrogen per day in Subject 3, when dietary nitrogen was derived exclusively from whole egg, since all stool nitrogen was endogenous assuming whole egg to be completely digestible.[1] The

linear relationship of dietary to fecal nitrogen is based on the assumption that as dietary protein decreased in the diets fed to these subjects, the dry weight of the diet remained relatively constant and the interrelationship of fecal nitrogen to the dry weight of the diet has been demonstrated by Mitchell.[22]

TABLE III. ENDOGENOUS FECAL NITROGEN AND ENDOGENOUS URINE NITROGEN IN FIVE CHRONIC RENAL INSUFFICIENT PATIENTS AND ONE HEALTHY SUBJECT*

	LOWEST OBSERVED FECAL NITROGEN		CALCULATED ENDOGENOUS FECAL NITROGEN		CALCULATED ENDOGENOUS URINARY NITROGEN (MG. PER BASAL CALORIE)
PATIENT	GRAMS/DAY	MG. PER BASAL CALORIE	GRAMS/DAY	MG. PER BASAL CALORIE	
1	1.18	0.651	1.06	0.590	2.91
2	0.74	0.604	0.69	0.565	3.69
3	0.58	0.513	0.60	0.520	3.27
4	1.10	0.666	0.96	0.585	3.16
5	0.99	0.611	0.84	0.516	3.39
Healthy subject	0.77	0.516	0.71	0.475	2.13

*Excluding proteinuric losses.

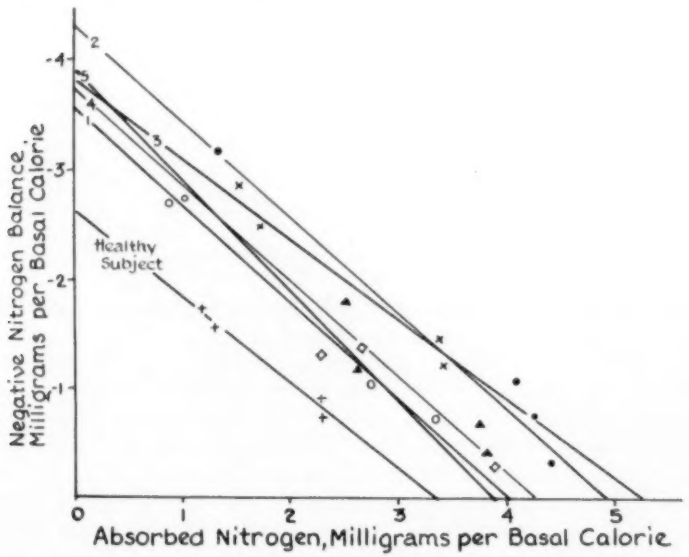

Fig. 1.—Negative nitrogen balance, excluding proteinuric losses compared to absorbed nitrogen for 5 chronic renal insufficient patients and one healthy subject. Key: ○ = Subject 1; ● = Subject 2; x = Subject 3; ▲ = Subject 4; ◇ = Subject 5; + = healthy individuals.

Subject 5, however, showed only a rough relationship between dietary and fecal nitrogen; consequently, the calculated value for endogenous fecal nitrogen in Subject 5 is subject to error. Nonproteinuric endogenous urinary nitrogen values, shown in Column 5 of Table III, were calculated by subtracting the endogeous fecal nitrogen losses from the y axis intercept of each curve in Fig. 1.

An estimation was made of the minimum nitrogen requirement based on data obtained from suitable periods in which negative nitrogen balance exceeded 10 per cent and proteinuric nitrogen losses were excluded.* Plotting the balance on the abscissa and the net absorbed nitrogen on the ordinate, a linear relationship of the data may be ascertained as shown by Mitchell[6] by the method of least squares (see Fig. 1). The data for Subject 3 are for a dietary protein source of 100 per cent whole egg; data for the remaining patients and the healthy subject are for a mixed diet.

The intercepts at nitrogen equilibrium and the slope of each curve are tabulated in Table IV. The "x" intercept represents the minimum absorbed nitrogen required for equilibrium; the "y" intercept shows the endogenous nitrogen metabolism of Folin. To test further the former observation, Subject 5 was studied on a diet calculated to meet nitrogen requirements according to the foregoing estimations, and nitrogen equilibrium (including proteinuric losses) was demonstrated.†

TABLE IV. COMPARISON OF MINIMUM NITROGEN REQUIREMENT AND ENDOGENOUS NITROGEN CATABOLISM IN RENAL INSUFFICIENT PATIENTS AND A HEALTHY SUBJECT*

PATIENT	X INTERCEPT MG. PER BASAL CALORIE	Y INTERCEPT MG. PER BASAL CALORIE	B SLOPE
1	3.95 ± 0.24	3.50	0.88
2	4.91 ± 0.23	4.34	0.88
3	5.20 ± 0.27	3.80	0.73
4	4.23 ± 0.33	3.75	0.88
5	3.84 ± 0.14	3.89	1.01
Healthy subject	3.32 ± 0.18	2.59	0.78

Y = Endogenous nitrogen metabolism.
X = Minimum nitrogen requirement.
*Excluding proteinuric losses.

Various attempts were made to influence the level of nitrogen metabolism in 4 patients. These observations are presented in Fig. 2. When 70 per cent of dietary protein was composed of cooked egg albumin in Subject 1 and when dietary protein was from mixed sources in Subject 3, there was no significant difference from data obtained for a mixed diet in Subject 1 or from the exclusively whole egg diet in Subject 3. The influence of amino acid supplements was studied. Supplementation with a mixture of the 8 amino acids deemed essential by Rose[23] resulted in data far to the right of that calculated from mixed, unsupplemented diets. Glycine resulted in a somewhat similar situation. Methionine seemed to be without influence in one patient. However, a mixture of L-amino acids, consisting primarily of L-leucine and L-isoleucine, showed a nitrogen sparing effect in Subject 3 and no special influence in Subject 1 at a level of 400 mg. supplementary nitrogen per day. When this

*Proteinuric nitrogen varied widely between patients and varied roughly with the dietary nitrogen in individual patients. Since proteinuric nitrogen is synthesized protein, subtraction of this loss from the nitrogen output gives nitrogen requirement data excluding proteinuric losses.

†Minimum nitrogen required for equilibrium, including proteinuric losses, expressed as net absorbed nitrogen (dietary nitrogen less total fecal nitrogen): calculated, 4.35 mg. nitrogen per basal calorie; observed, 4.41 mg. nitrogen per basal calorie; balance: calculated, 0; observed, −0.89 Gm. nitrogen per day, −9.5 per cent, −0.58 mg. nitrogen per basal calorie. (See Table II, Patient 5, last two periods.)

level was increased to 1,000 mg. nitrogen per day and, in addition, 400 mg. gelatin nitrogen was given as a supplement, no special influence on nitrogen metabolism was demonstrated in Patient 5. (Table II.)

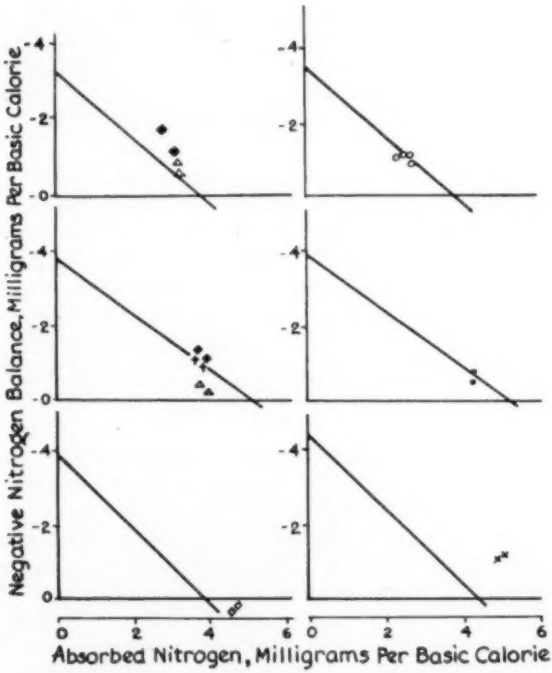

Fig. 2.—The effect of alterations in the amino acid pattern of dietary nitrogen as compared to a mixed diet of natural foods. Curves are derived from the data of Fig. 1. Key: ○ = 70 per cent dietary nitrogen derived from purified egg albumin; ● = dietary nitrogen derived from a mixed diet compared to curve for diet of 100 per cent whole egg; ✦ = 400 mg. glycine nitrogen per day; △ = 400 mg. nitrogen per day from a mixture described in Table II under supplement "C"; + = 400 mg. L-methionine nitrogen per day; x = 1,134 Gm. nitrogen from mixture of 8 amino acids described in Table II; ✧ = 1,000 mg. nitrogen from mixture described in Table II, under supplement "C," plus 400 mg. gelatin nitrogen per day.

DISCUSSION

The primary clinical derangement in each of the 5 patients in contrast to the normal subject was chronic renal insufficiency. The extent and manifestations varied from few symptoms (Subject 3) to complete disability (Subject 2). Generally, as the NPN fell the patient improved clinically. The metabolic abnormalities likewise were variable and not in direct accord with the clinical state. The sickest patient, clinically (Subject 2), was not the most abnormal in terms of nitrogen balance; the minimum nitrogen need does not directly parallel the clinical state. It does, however, remain unequivocally elevated when compared to the healthy subject and to the data on normal subjects in the literature.

In all patients and in the healthy subject, nitrogen equilibrium (balance to with ± 10 per cent of ingested nitrogen) was attained. The high nitrogen needs of the chronic renal insufficient patients cannot be explained by proteinuria alone. Statistical comparison revealed that the values of Subject 1, 3.95 ± 0.24 mg. nitrogen per basal calorie, and Subject 5, 3.84 ± 0.18 mg. nitrogen per basal calorie, differ significantly from the healthy subject, t = 3.63 and 4.27, respectively ($p = <0.01$). Farr[24] demonstrated an increased nitrogen requirement for children with nephrosis. Based on urinary nitrogen measurements, Olbrich[15] likewise demonstrated an increased nitrogen requirement in chronic kidney disease.

The observed minimal nitrogen requirement of 3.32 ± 0.18 mg. per basal calorie for the healthy subject is equivalent to 2.07 mg. per basal calorie when requirements for growth are excluded and the biologic value of the dietary protein (derived from the slope of retrogression lines of nitrogen balance on absorbed nitrogen intake) is mathematically increased from the observed 78 to 100 per cent. This is in agreement with other reported minimum nitrogen requirements,[1, 23] although some of the data by others were obtained on an intake of 50 to 60 calories per kilogram compared to 30 calories per kilogram in this study. These patients could not consistently consume a diet of higher caloric content.

The explanation for the increased protein requirement of the renal insufficient patient is not evident from the data available on the subject. Either a general reduction in the efficiency of protein utilization or an increased requirement for a given amino acid could have produced the observed differences from the normal subject.* The data presented here on the effect of glycine and the 8 essential amino acids are thought to minimize the first possibility. Investigations of the second possibility did not produce clear-cut results. The amino acid, L-methionine, was thought to be the most likely prospect as a protein sparer from the experience of others[25-28] with its influence on nitrogen metabolism together with the observation of excessive urinary losses of sulfur in chronic nephritis by Grabfield.[30-32] Thus, its effect was investigated first, but no significant influence was demonstrated. The isoleucine, leucine, and methionine mixture seemed to exert a definite, though small, nitrogen sparing effect. It has been shown that all the essential amino acids of Rose exert a nitrogen sparing effect in normal animals except valine, tryptophane, and phenylalanine.[33] The experiments of Elvehjem[29] suggest that in any state in which nitrogen intake is inadequate, there is a most limiting amino acid. Supplementation of the diet with the limiting substance improves the diet out of proportion to the supplementary nitrogen, but a supplement of a nonlimiting amino acid accelerates the rate of nitrogen metabolism, and, as a result, the limiting factor is more apparent and the diet relatively less adequate than before.

The accelerated destruction of hemoglobin, which has been shown to occur in renal insufficient patients,[34] may be a major factor in the greater

*No distribution of nitrogen excreted in the urine was made. However, paper chromatograms indicated that there was no significant aminoaciduria in these subjects.

nitrogen requirement of these patients. There is also the possibility that the survival of other cells of the body undergoing continuous and extensive regeneration, such as the white blood cells and intestinal epithelium, may be altered in renal insufficiency.

These data provide a basis for estimating the approximate protein requirement of chronic renal insufficient patients as compared to that of the healthy subject similarly maintained. The minimal protein requirement from a mixed diet, including a factor for growth, is shown in Table V and is based on the data obtained on these 5 patients and the normal individual.

TABLE V. COMPARISON OF NITROGEN REQUIREMENT OF A RENAL INSUFFICIENT PATIENT AND A HEALTHY SUBJECT

	FIVE PATIENTS (RANGE)	NORMAL SUBJECT
mg. Nitrogen/basal calorie	4.35 - 5.70	3.80
Gm. Protein/Kg.	0.54 - 0.70	0.49
Gm. Nitrogen/Kg.	0.086- 0.11	0.079
Gm. Nitrogen/Surface Area, M^2	3.90 - 4.30	3.20
Gm. Protein/Surface Area, M^2	24.4 -26.9	20.0

SUMMARY

Nitrogen balance studies were conducted on 5 renal insufficient patients at several levels of protein intake. Similar observations were made on a healthy individual. The data indicate that renal insufficient patients manifesting slight to moderate azotemia had a higher than normal endogenous nitrogen metabolism and required a higher protein intake for nitrogen equilibrium than did the normal subject on the same diet. By means of retrogression curves, it was possible to predict the protein required for nitrogen equilibrium. The actual protein requirement for a given patient could not be predicted from any of the clinical measurements applied to these patients. Limited amino acid supplementation of the basic protein restricted diet failed to demonstrate any effect on the nitrogen requirement of these patients.

We are indebted to Professor H. H. Mitchell for his helpful comments concerning this paper and wish to thank Mrs. Ruth M. Strickland, dietitian, for her collaboration and contribution to these studies.

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THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ABNORMAL GLOBULINS CONTAINED IN HEAT STERILIZED HUMAN PLASMA

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THE high incidence of serum hepatitis following intravenous administration of pooled plasma to human beings[1, 2] has stimulated the search for, first, effective and innocuous plasma substitutes[7] and, second, a satisfactory method for sterilizing pooled plasma.[3, 4] Both aims have been only partially successful.[1, 5-7]

In 1948, Gellis and associates[8] reported that the virus of serum hepatitis can be inactivated by heating for 10 hours at 60° C. a mixture containing 40 ml. of 25 per cent human serum albumin and 10 ml. of plasma known to be icterogenic. Unfortunately, when this method is applied to serum or plasma, a coagulum is formed and the electrophoretic properties are altered.[15] Even heating of pooled plasma at 60° C. for only 4 hours causes the plasma to become turbid, forming a precipitate, and is insufficient to destroy the virus.[9] Recently, Mulford and his co-workers[10, 11] reported a method for forming a heat-stable human plasma protein solution. This involved the preliminary separation of heat-labile proteins by cold alcohol precipitation.

The heated plasma used in our study was obtained from a commercial source* and was prepared by a different but relatively more simple method. Because of its potential clinical usefulness, we decided to investigate the metabolism of the globulins from heated plasma which appear abnormal by electrophoresis.

MATERIALS AND METHODS

The heated plasma was prepared from blood collected with a sodium citrate anticoagulant. The plasma was separated and stored frozen until a sufficient amount had accumulated to make up the lot. It was then thawed, clarified, and irradiated, using the latest modification of the Oppenheim-Kettering apparatus; the plasma was then defibrinated by the addition of sufficient calcium chloride to overcome the effects of the citrate present. The clot which formed was removed, and the liquid was passed through a set of clarifying filter pads. Next it was passed through a sterilized ion exchange column containing resin in the sodium cycle to reduce the calcium content to the normal value. Sterile dextrose was then added to make a final concentration of 5 per cent, and the preparation was passed through clarifying and

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sterilizing filter pads. It was then immediately filled into its final containers, stoppered, and heated for 10 hours at 60° C. The lot which we used was processed in October, 1955, and was stored at room temperature until used.[12] The unheated plasma, also, was obtained from blood collected with a sodium citrate anticoagulant.

The globulins of both heated and unheated plasma were separated from the albumin fraction using the Spinco Model CP Continuous Flow Paper Electrophoresis Cell with a 0.02 M barbital buffer at pH 8.6. The globulin fractions were collected and pooled. The pooled globulins represented all the globulins originally present except for an estimated one-half of the alpha-1 fraction. This amounted to about 2 per cent of all the globulins. By suitably adjusting buffer flow, the protein concentration of pooled globulins could be kept sufficiently high to be used for labeling without removing the buffer and without further concentration. Approximately 20 mg. each of heated and unheated globulins were labeled with I^{131} by the technique of Pressman and Eisen,[13] except that dialysis was not done to remove the unbound iodine. Unbound iodine was removed by precipitation of the globulins with saturated ammonium sulfate. The precipitate was centrifuged at -5° C. for 5 minutes and the supernatant decanted. The precipitate was redissolved in normal saline and reprecipitated two times. Then 5.0 ml. of heated plasma was added to the labeled heated globulins and 5.0 ml. of unheated plasma to the labeled unheated globulins as carrier. In both final preparations, approximately 95 per cent of the I^{131} present was precipitable by trichloroacetic acid, and about 8 per cent of the originally added radioiodine was protein bound. The activity of the final preparations in millicuries per milliliter of protein solution was determined with a calibrated electroscope. By precipitation with ammonium sulfate, the total time necessary to remove the unbound iodine was about 45 minutes.

Both final preparations of labeled heated and unheated globulins contained about 4 to 6 atoms of I^{131} per molecule of protein. We did not attempt to determine the molecular weights of the heated globulins and used published data[14] for normal globulins to determine the average number of I^{131} atoms per molecule. The iodinated globulins were passed through a Berkfeld filter and determined to be pyrogen-free by rabbit inoculation and sterile by culture before administration.

For paper strip electrophoresis, a Durrum cell was used with a barbital buffer of ionic strength 0.075 and pH 8.6. Twenty to fifty lambda of protein solution were applied to Whatman No. 3 paper strips and electrophoresis was done for 16 hours. The proteins were stained with bromphenol blue. Protein fraction concentrations were determined by dye density readings using an Analytrol* with a B-3 cam and blue glass filters. A Nuclear-Chicago D-47 gas flow counter and a C-100 Actigraph were used to determine the distribution of the iodinated globulins in sera.

About 200 μc of labeled heated globulins were given intravenously to each of 3 patients and about 200 μc of labeled unheated globulins to each of 2 patients. None of the patients were febrile or had any obvious abnormality of protein metabolism. All were hospitalized during the period of study and received 10 to 30 drops of a saturated solution of potassium iodide daily, starting 24 hours prior to receiving the iodinated globulins.

Blood samples and 24-hour urine specimens were collected daily from each patient. The blood was allowed to clot, and, after centrifugation, 3 ml. aliquots were removed for counting. For determination of protein-bound I^{131} in the urine, 1.0 ml. of normal plasma was added, as a carrier, to 2.0 ml. of urine.

All counting was done in a well counter, using a thallium-activated sodium iodide crystal and a pulse height analyzer. Background counts were approximately 20 per minute. All samples were counted for sufficient time to give less than a 2 per cent counting error.

Protein-bound I^{131} was determined by the method of Freedberg and associates[20] using 3.0 ml. of sera and 2.0 ml. of urine with 1.0 ml. of blood bank plasma added.

The density curves and radioactivity distribution curves obtained from the filter paper strips could be accurately compared because each paper strip was marked with a lead pencil,

*Spinco Division, Beckman Instruments, Inc., Belmont, Calif.

the tip of which had been immersed momentarily in a solution containing I^{131}. This produced a small mark on each density and radioactivity curve which could be superimposed.

Thirty-four days after completing the study, 15-minute plasma volumes, using I^{131}-labeled human serum albumin,* were done on one patient of each group. These were compared with the plasma volumes found using iodinated globulins.

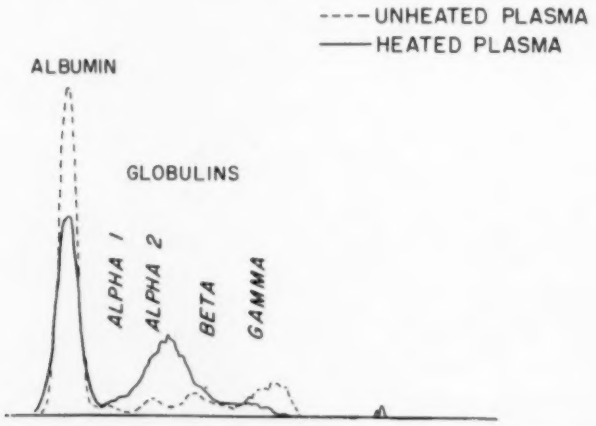

Fig. 1.—Comparative electrophoretic patterns of heated and unheated plasma. The small marks on the extreme right represent pencil marks which could not be superimposed exactly because the samples were from two different runs.

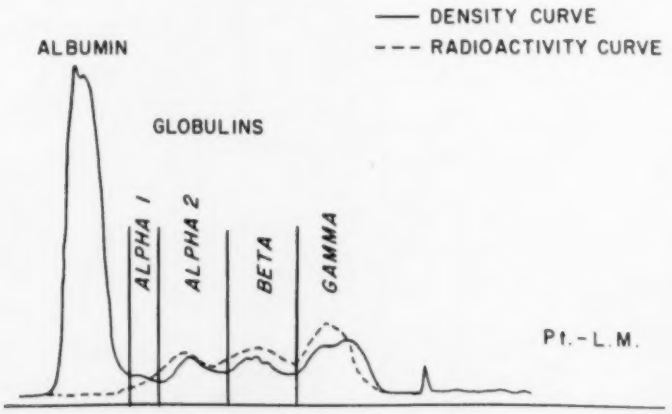

Fig. 2.—This illustrates the density and radioactivity distributions of labeled unheated globulins. One drop of the labeled globulin preparation was added to 0.5 ml. of normal sera and the resulting mixture subjected to electrophoresis.

RESULTS

Compared with normal plasma, the heated plasma we used contained relatively less albumin and much more alpha-2 and beta globulin fractions (Fig. 1).

*RISA, Abbott Laboratories, North Chicago, Ill.

On electrophoresis, the albumins migrated to their usual position and were sharply demarcated from the globulins. However, the globulins did not separate into the fractions usually seen on electrophoresis of unheated plasma.

The labeled globulins from unheated plasma migrated on electrophoresis in the same manner as the unlabeled globulins, with close approximation of the density and radioactivity distribution curves (Fig. 2).

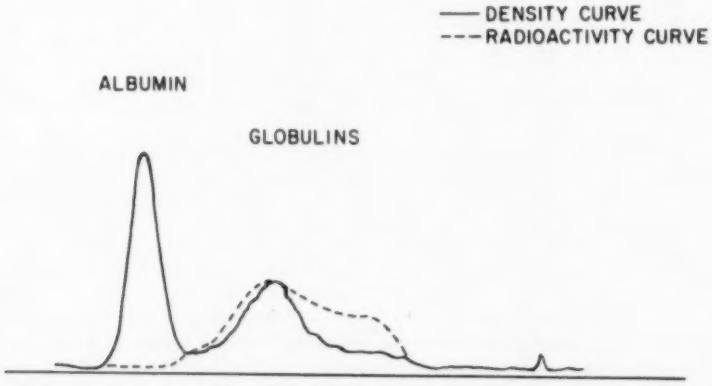

Fig. 3.—This illustrates the density and radioactivity distributions of labeled heated globulins. One drop of the labeled globulin preparation was added to 0.5 ml. of heated plasma and the mixture subjected to electrophoresis.

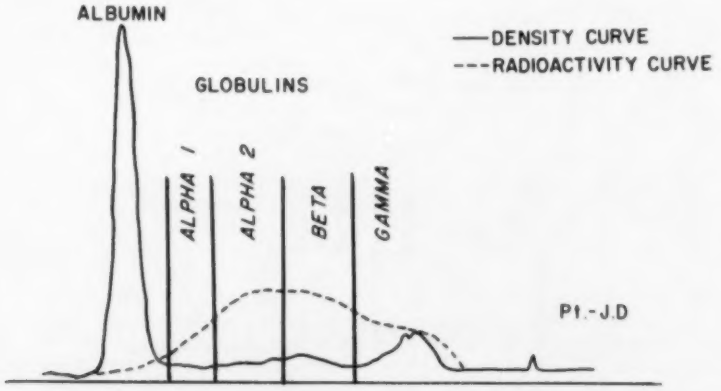

Fig. 4.—The radioactivity and density distributions were made from the 15-minute serum specimen of a patient who received labeled heated globulins.

The labeled heated globulins also migrated on electrophoresis in a similar manner as the unlabeled heated globulins (Fig. 3). However, the density and radioactivity curves did not closely approximate each other in vitro (Fig. 4). The wider distribution of radioactivity in Fig. 4 as compared with Fig. 3 is due to the use of a larger slit over the window of the gas flow counter.

I^{131} activity was measured in the sera of all patients for 30 days. The decline in radioactivity was plotted on semilogarithmic paper, using the 15-minute samples as a 100 per cent value (Fig. 5). Protein-bound I^{131} determinations were made on every third serum specimen, and all showed more than 92 per cent of the activity to be protein bound. Protein-bound I^{131} determinations were made on all urine specimens collected during the first week, and no protein-bound I^{131} was found in any specimen.

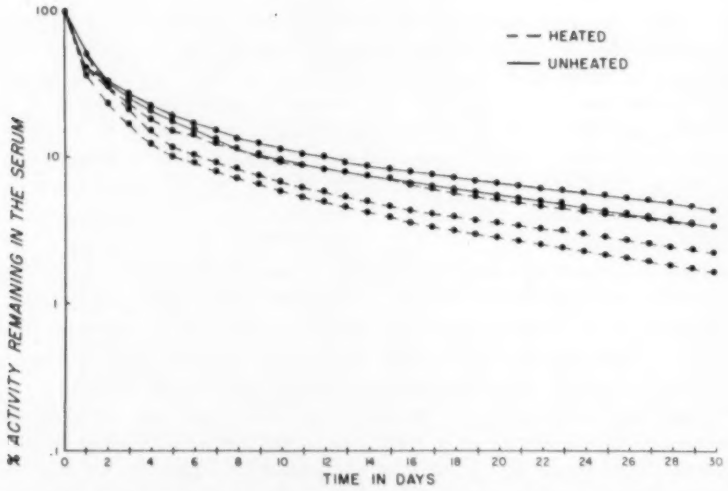

Fig. 5.—This illustrates the serum radioactivity decay curves of 2 patients who received labeled unheated globulins and 3 patients who received labeled heated globulins intravenously.

The half-times obtained for heated globulins were 11.62, 14.75, and 13.71 days, and for unheated globulins were 14.71 and 13.54 days. On inspection of Fig 5, it appears that there was a more rapid initial disappearance of radioactivity from the serum of patients who received the labeled heated globulins.

The plasma volume of the patient who received heated globulins was determined to be 4,204 ml. using I^{131}-labeled human serum albumin and 4,738 ml. using I^{131}-labeled globulins. The patient who received unheated globulins showed a plasma volume of 2,577 ml. using I^{131}-labeled human serum albumin and 2,695 ml. using I^{131}-labeled globulins.

DISCUSSION

The lack of close approximation of the radioactivity distribution and density curves of labeled heated globulins in vitro could be due to (1) further alteration of the globulins by the labeling or separation procedures or (2) nonuniform labeling. We could find no conclusive evidence that the labeling procedure altered the electrophoretic mobility of the globulins. When the procedure was repeated without I^{131} present, the density distributions were the same as for the original globulins, except that the globulins migrated a shorter distance, even when the pH was brought to 7 by adding 0.1 N NaOH. This effect could have

been due to the added sulfate and sodium salts. However, any effect of these ions could not explain the in vivo distribution of radioactivity, which closely resembled that of the in vitro distribution.

It is probable that heated plasma contains denatured globulins which remain in solution. Observations in this laboratory indicate that denatured proteins iodinate more readily than natural, unaltered proteins. Thus, the discrepancy in the radioactivity and density curves could be due to higher labeling by denatured proteins.

The plasma volumes obtained using I^{131}-labeled human serum albumin, heated globulins, and unheated globulins were in fairly close agreement. This indicated that no large amount of the globulins were lost during the first 15 minutes after injection. The patient who received heated globulins was hospitalized for emphysema and lost 12 pounds in weight between the time that his plasma volumes were determined using heated globulins and I^{131}-labeled human serum albumin. Thus, his 11.2 per cent lower plasma volume found using I^{131}-labeled human serum albumin may, in part, have been due to an actual reduction of his blood volume.

The turnover rates we obtained represent that of the longest lived components present in the globulins and are within the normal range reported for gamma globulins.[16, 17] All normal plasma proteins do not have the same turnover rates.[18, 19] Since gamma globulins are heat labile, the long-lived components of heated plasma must be largely globulins other than gamma globulins.

Although their disappearance rates are approximately the same, heated globulins appear to equilibrate at a lower level than unheated globulins. Our results suggest that about 30 per cent of the heated globulins are metabolized much more rapidly than the unheated globulins. However, the difference could be due, at least in part, to the removal, by precipitation during heating, of the longer lived but heat-labile components. Then, the remaining globulins would contain a relatively greater quantity of rapidly metabolized proteins. Since we found no protein-bound I^{131} in the urine, there is no evidence of loss of globulins by this means.

Although some initial rapid catabolism of heated globulins may occur, it is obvious that the majority persist in the serum for several days. We were able to follow, with good counting rates for 40 days, the serum activity of one patient who received heated globulins. This property would seem to be quite desirable if heated plasma were to be used as a plasma volume expander.

SUMMARY

1. The electrophoretically abnormal globulins contained in heated human plasma were separated from albumin by paper curtain electrophoresis. After labeling with I^{131}, the globulins were given intravenously to 3 patients. The globulins from unheated plasma were separated and labeled with I^{131} by the same techniques and given intravenously to two patients.

2. The serum activities of all patients were followed for a minimum of 30 days. Disappearance rates of the longest lived components of both heated and unheated globulins were within the normal range reported for gamma globulins.

3. An estimated one-third of the heated globulins disappeared from the serum at a more rapid rate than the unheated globulins. Possible explanations for this are discussed.

4. It appears from this study that globulins from heated plasma persist in the serum after intravenous administration for a sufficient time to be useful as globulins for plasma volume expansion.

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PENICILLIN RESISTANCE OF STAPHYLOCOCCI CARRIED BY INFANTS AND YOUNG CHILDREN

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PERSONS carrying penicillin-resistant staphylococci apparently are becoming increasingly numerous in the general population. Although Dowling, Lepper, and Jackson[1] observed some tendency for hospital patients to lose their resistant strains after 7 weeks at home, other investigators have reported disturbingly high carriage among persons not recently hospitalized. For example, Rountree and Rheuben[2] observed nasal carriage of resistant strains among blood donors to increase from 4 per cent in 1949 to 6.5 per cent in 1954, and to 13 per cent in 1955. In 1954, Fusillo, Roerig, and Ernst[3] reported 17.8 per cent of blood donors to carry resistant strains in their throats and 6.7 per cent on their skins.

The above observations apply to the carrier state of adults, and do not estimate that of infants and young children. In many hospitals today 90 per cent or more of the newborn infants acquire penicillin-resistant staphylococci while in the nursery[4] and some retain the nursery strains for at least a year.[5] This implies that children born in recent years may harbor resistant staphylococci with even greater frequency than the present generation of adults. A survey was conducted to investigate this possibility.

MATERIALS AND METHODS

Nose and throat cultures were obtained on 208 healthy children, aged 15 months to 5 years, at the time they attended or accompanied their siblings to the Well Baby Clinic. For comparison, cultures were also taken on 180 healthy older children who attended the orthodontic and dental hygiene clinic. They were between 10 and 18 years of age and, therefore, born before the extensive use of penicillin in hospitals.

The child's experience with penicillin therapy was recorded at the time cultures were collected. Since the older children were seldom accompanied by their mothers, the therapeutic information may be less reliable for this group than for the younger age group, but each instance of real doubt was noted.

The swabs were plated immediately on brain heart infusion agar and incubated for 48 hours. Cream or golden pigmented colonies were tested for coagulase by the slide method, and only those producing it are cited in this report. One colony per culture was tested for sensitivity to disks containing 10 units of penicillin. Strains which showed inhibition zones of 3 mm. or less were considered resistant while those with zones 8 mm. or over were recorded as sensitive. Zones of intermediate width were not observed.

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RESULTS

To determine whether the presence of resistant strains might be related to the site of carriage and to permit comparisons with the surveys which report nasal or throat cultures, the nasal and throat culture results are considered independently as well as collectively in Tables I, II, and III.

Incidence of Coagulase-Positive Staphylococci.—Nasal carriage (Table I) increased from 31 per cent among infants aged one year to 70 per cent among children between 10 and 12, and then fell to about 25 per cent after puberty. Such differences between the age groups sampled are statistically significant and unlikely to have occurred by chance ($\chi^2 = 27.24$; $P = < 0.001$). Moreover, the figures for the first 12 years of life correspond closely with those of Cunliffe[6] who reported an increase in nasal carriage from 23 per cent in the first year to 65 per cent between the ages of 8 and 10.

Throat carriage (Table I) remained at a fairly constant level among children of both age groups. Although it appeared to decline slightly after the twelfth year, similar differences could have occurred by chance in samples of this size.

TABLE I. CARRIAGE OF COAGULASE-POSITIVE STAPHYLOCOCCI

AGE IN YEARS	TOTAL NO. CHILDREN	PER CENT CARRYING STAPHYLOCOCCI IN		
		NOSE	THROAT	EITHER OR BOTH
1- 2	86	31	63	68
2- 3	71	37	61	69
3- 4	29	55	62	73
4- 5	22	55	55	68
10-12	20	70	55	75
12-14	86	50	47	64
14-16	57	23	44	54
16-18	17	29	35	51

The combined nasal-throat carrier rate (Table I) was similar in children under 12 years of age. After the age of 12 it appeared to diminish with increasing age and this inverse relationship is not likely to have occurred by chance ($\chi^2 = 8.68$; $P = 0.04$). Staphylococci in the throat were responsible for the relatively high combined carrier rate of children under 3 years, indicating that throat cultures assess the carrier state more accurately than nose cultures at this age.

Incidence of Penicillin-Resistant Strains.—The number of children with resistant staphylococci in the nose, throat, or both is shown in Table II. Forty-five per cent of the total group of children under 5 yielded resistant strains from either or both sites, while this was true of 18 per cent of the group aged 10 to 18 years. This difference is highly significant and is not likely to have occurred by chance ($\chi^2 = 30.64$; $P = < 0.001$).

The contrast between these two groups cannot be explained by their different carrier rates because a similar discrepancy was obtained when only those children with coagulase-positive staphylococci were considered (Table

III). Sixty-four per cent of the younger children whose cultures were positive had resistant strains, whereas 32 per cent of the older children yielded them. The incidence of resistant strains in nasal cultures of children under 3 was high (Table III) despite the fact that the nasal carrier rate is low at this age (Table I).

TABLE II. RESISTANT STRAINS AMONG THE TOTAL NUMBER OF CHILDREN

AGE IN YEARS	TOTAL NO. CHILDREN	PER CENT CARRYING RESISTANT STAPH. IN			
		NOSE	THROAT	EITHER OR BOTH	
1- 2	86	19	43	48	
2- 3	71	18	38	42	45
3- 4	29	10	38	45	
4- 5	22	27	18	41	
10-12	20	15	10	25	
12-14	86	9	10	16	18
14-16	57	4	21	21	
16-18	17	6	12	12	

TABLE III. INCIDENCE OF RESISTANT STRAINS AMONG CHILDREN CARRYING COAGULASE-POSITIVE STAPHYLOCOCCI

AGE IN YEARS	PER CENT CARRIERS WITH RESISTANT STAPH. IN			
	NOSE	THROAT	EITHER OR BOTH*	
1- 3	55	66	66	64
3- 5	32	50	61	
10-14	18	22	27	32
14-18	17	46	37	

*While the majority of children whose nasal and throat cultures were both positive yielded strains of similar sensitivity from both sites, exceptions were found. Nine had a resistant strain in the nose and a sensitive one in the throat, while the reverse combination was found in 16.

Although resistant strains appeared to be somewhat less frequent in the nasal cultures of children aged 3 to 5 as compared with those aged 1 to 3 (Table III) this difference is not statistically significant. Similarly, the higher incidence of resistant strains among throat carriers aged 14 to 18, as compared to those aged 10 to 14 could have been due to chance.

TABLE IV. RELATIONSHIP BETWEEN PENICILLIN EXPERIENCE AND CARRIAGE OF RESISTANT STRAINS

AGE IN YEARS	1-5		10-18	
PENICILLIN EXPERIENCE	PER CENT OF CARRIERS WHOSE STRAINS WERE			
	RESISTANT	SENSITIVE	RESISTANT	SENSITIVE
Yes	42	46	55	59
No	53	48	36	35
Uncertain	5	6	9	6

Contrary to expectation, there was no evidence that previous penicillin experience necessarily accounted for the resistant staphylococci obtained from either age group (Table IV). Sensitive strains occurred as frequently as resistant strains among children who had received penicillin. Conversely, children who never had been given penicillin yielded resistant and sensitive strains with about equal frequency.

In view of the high incidence of resistant staphylococci among the younger children, the older children who yielded resistant strains might have acquired them from their younger siblings. However, the records indicated that this had not been a general rule (Table V). Sensitive strains were somewhat more common than resistant strains among those who had siblings under the age of 10, whereas the reverse was true of those whose siblings were over 10 years old.

TABLE V. RELATIONSHIP BETWEEN CARRIAGE OF RESISTANT STRAINS AND SIBLINGS UNDER OR OVER 10 YEARS OF AGE

SIBLINGS	PER CENT CHILDREN AGED 10 TO 14 WITH STAPH.	
	RESISTANT	SENSITIVE
Some under 10	36	52
All 10 or over	58	33
None	6	15

DISCUSSION

This survey demonstrates that young children are now carrying penicillin-resistant staphylococci with an alarming frequency. It is probable that the majority are of nursery origin because (1) staphylococci acquired by newborn infants are known to be carried for long periods of time; (2) children born prior to the extensive use of penicillin in hospitals do not exhibit this high incidence of resistant strains, and (3) carriage of resistant staphylococci was not correlated with penicillin experience in this study nor has such a correlation been apparent in surveys made on adults.[2, 7] Rountree, Freeman, and Barbour[7] found carriage of resistant strains among blood donors to be more closely associated with previous hospitalization and/or penicillin therapy than with penicillin therapy alone.

It is evident from this data that the pediatric value of penicillin for staphylococcal infections has greatly diminished. That staphylococcal infection is often endogenous in both infants and adults has been substantiated by both serologic and phage typing methods.[8-11] If the present generation of young children continue to carry their resistant strains indefinitely as has been suggested,[5] many staphylococcal infections in the general population will fail ultimately to respond to penicillin. This possibility, together with the marked ability of staphylococci to develop resistance to many other antibiotics, suggests that antibiotics may have little value eventually in treating staphylococcal infections. Numerous investigators have warned that the future value of antibiotics in staphylococcal infection depends on their more discriminate usage, particularly in hospitals. Their value may also depend upon preventing newborn infants from becoming colonized with resistant strains during their stay in the nursery.

SUMMARY

A survey was conducted to determine the incidence of penicillin-resistant staphylococci among children born in recent years as compared to those born

prior to extensive hospital use of penicillin. Forty-five per cent of the children under 5 were found to harbor penicillin-resistant staphylococci, in contrast to 18 per cent of the children between 10 and 18 years of age.

Penicillin resistance of the staphylococci obtained was independent of previous penicillin experience. It is postulated that the majority of resistant strains recovered from the younger children are of hospital nursery origin. The future value of antibiotics in treating staphylococcal infection may depend, in part, upon preventing newborn infants from acquiring resistant strains while in the hospital nursery.

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THE ALKALINE PHOSPHATASE CONTENT OF HUMAN LEUKOCYTES FOLLOWING MARROW SUPPRESSION BY NITROGEN MUSTARD

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THE early application of the histochemical techniques of Gomori[1] and Takamatsu[2] to the study of human blood and marrow cells promptly disclosed the presence of an alkaline phosphatase in the polymorphonuclear leukocytes.[3-7] Biochemical confirmation and quantitation soon followed.[8] Although precise cellular and intracellular localization of the enzyme by histochemical methods is made difficult by diffusion artefacts,[9] it is now generally agreed that for marrow and blood only the myeloid elements and reticulum macrophages give positive staining reactions for alkaline phosphatase. The adult neutrophil has been recognized as the major site of the enzyme activity, but little has been said concerning the alkaline phosphatase content of its precursor elements. Wachstein[3] commented on the difficulty of staining marrow preparations for alkaline phosphatase but reported nevertheless a much larger number of positive cells in the marrow than in the peripheral blood. The positive cells were defined simply as leukocytes and no definite statement was made concerning the level of cellular differentiation. He further suggested that the alkaline phosphatase activity of the leukocyte was acquired in the bone marrow. Rheingold and Wislocki[4] demonstrated alkaline phosphatase in the neutrophil, metamyelocyte, and myelocyte and thought it to be most abundant in the latter. Our histochemical experience,[10] based on the use of sodium alpha naphthyl acid phosphate as substrate, showed that the major alkaline phosphatase activity was present in the band and segmented neutrophilic polymorphonuclear leukocytes with only trace activity in the late myelocytes. Myeloblasts and promyelocytes gave negative staining reactions. Wiltshaw and Moloney[11] using a modified Gomori technique found the alkaline phosphatase confined to the adult and band neutrophil in the marrow. Although somewhat contradictory, the cytochemical evidence to date suggests that the polymorphonuclear leukocyte is much richer in alkaline phosphatase than its precursors.

Thus, if alkaline phosphatase is present primarily in the more fully differentiated members of the myeloid cells, then enzyme concentration may be

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TABLE I. ABSOLUTE DATA OBTAINED ON 4 PATIENTS BEFORE AND AFTER NITROGEN MUSTARD ADMINISTRATION

DAYS AFTER NITROGEN MUSTARD	PERIPHERAL WHITE COUNT (PER MM.^{3})				PERIPHERAL POLYMORPHONUCLEAR COUNT (PER MM.^{3})				ALKALINE PHOSPHATASE (MG. P PER 10^{10} POLYS PER HR.)			
	R. H. 1	E. F. 2	L. H. 3	P. L. 4	R. H. 1	E. F. 2	L. H. 3	P. L. 4	R. H. 1	E. F. 2	L. H. 3	P. L. 4
0	13,900	34,400	9,700	8,700	11,120	27,176	7,372	6,525	135.5	83.3	83.0	68.4
2	17,100	25,500		7,250	13,338	22,440		5,728	152.5	124.2		41.8
4	7,350		7,850		5,807		6,123		146.3		69.7	
5				5,150				4,175				98.7
6		16,100	7,450			13,524	5,211			83.7	88.8	
7	3,750			3,950	3,038			3,081	183.9			109.9
8		13,400	5,100			12,194	4,029			190.2	70.9	
9	1,900			3,300	1,273			2,574	175.8			92.5
10		8,950				7,160				113.4		
11	1,350		6,550		743		3,668		182.1		168.7	
12				3,350				2,513				183.6
13		2,025	5,750			812	2,680			417.8	157.2	
14	2,850			2,900	1,482			1,711	328.1			112.2
15		4,375				2,645				210.2		
16	4,100			2,400	2,706			1,152	297.6			186.5
17		7,150				4,934				143.2		
18	4,850		6,650		2,813		4,522		210.5		166.0	
19				5,100				3,010				142.1
20		6,850	9,250			5,069	6,198			198.2	162.3	
21	3,800			3,000	2,432			1,650	256.3			103.3
22		10,800	8,150			7,884	4,890			130.9	179.6	
23	3,850				2,118				285.6			
24		17,050				11,935				158.9		
25	5,500		8,500		2,915		5,350		219.4		146.4	
27		22,150	11,150	3,500		16,600	6,579	2,170		155.4	135.9	75.0
28	7,050				3,878				319.2			
29		23,200	7,950			17,400	4,929			158.4	178.6	
30				7,050				3,455				53.9
31	8,900	31,150			5,162	25,232			160.4	154.6		
33			8,000				5,760				81.3	
35	10,300	46,850			7,313	32,264			165.6	117.5		
36			9,350	8,250			5,891	5,115			91.1	141.1
39	13,600		12,500		10,608		8,435		55.5		63.0	
43	13,350				9,345				122.3			

Patients R. H. and P. L. (bronchogenic carcinoma) received a total dosage of 0.6 mg. per kilogram of body weight.
Patients E. F. (Hodgkin's disease) and L. H. (bronchogenic carcinoma) received a total dosage of 0.4 mg. per kilogram of body weight.

a biochemical expression of cell maturity. To test this hypothesis, temporary myleosuppression was induced with nitrogen mustard with the aim of achieving, during recovery from the hypoplasia, an initial neutrophil population, biochemically immature. Hence, a fall in leukocyte alkaline phosphatase content was anticipated.

MATERIALS AND METHODS

Three patients suffering from carcinoma of the lung and one patient with advanced Hodgkin's disease were the subjects of this study. Nitrogen mustard (methyl-bis [β-chloroethyl] amine hydrochloride) was administered for therapeutic purposes. Two patients received a total of 0.4 mg. per kilogram of body weight and 2 patients 0.6 mg. per kilogram of body weight intravenously in 2 equal doses. Separation of the leukocytes and the determination of alkaline phosphatase were carried out as previously described.[12] Total leukocyte and polymorphonuclear counts were determined on the same samples drawn for the alkaline phosphatase measurements. Values of the enzyme activity were expressed in terms of milligrams of phosphorus released from the substrate in one hour by 10^{10} polymorphonuclear leukocytes (i.e., all segmented forms). No cells younger than metamyelocytes were encountered in the blood of the subjects during the entire period of the study.

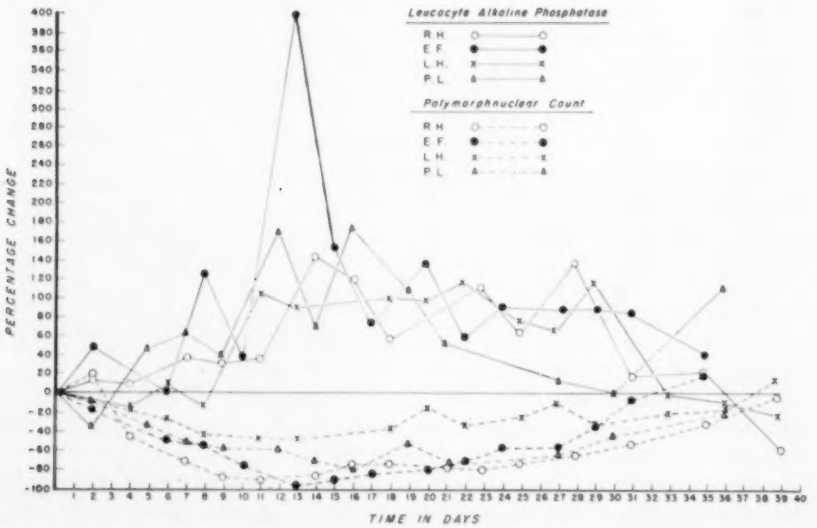

Fig. 1.—Per cent variation from pretreatment levels of leukocyte alkaline phosphatase and polymorphonuclear count.

RESULTS

The pertinent data derived from this study are shown in Table I. Fig. 1 represents a plot of this data in terms of per cent variation from the initial pretreatment levels. At first glance, the familiar response to nitrogen mustard is apparent. All 4 subjects displayed a leukopenia, a result primarily of granulocytopenia, which became most prominent between the eleventh and

sixteenth days after the administration of the drug. The depth and speed of the cell count depression were independent of the inital cell numbers or the dosage of the drug administered. The cells then stabilized at somewhat higher levels for the next 10 or so days; then they increased rapidly to return to pretreatment values. This sequence of events has been adequately described in the past and the curves found in this study are essentially identical to those shown by Wintrobe.[13] It is interesting to note that the change in the peripheral blood is due primarily to a change in the number of polymorphonuclear cells.

The alkaline phosphatase values of the polymorphonuclear cells following nitrogen mustard administration showed striking changes in opposite direction to the counts. The initial pretreatment values of the enzyme were high and were probably due to the inflammatory changes accompanying the patients' basic disease processes. During the first few days after treatment there was little change in the enzyme content of the polymorphonuclear cells, but as the counts began to fall, increasing values of the enzyme were obtained and then a sharp rise followed (to at least 100 per cent of pretreatment levels) as the low point in the polymorphonuclear cell count was reached. The alkaline phosphatase content of the cells then tended to stabilize at these high levels for the next 10 or so days, that is, during the period of relative cell count stability. A sharp decline in the alkaline phosphatase content of the cells to pretreatment values then followed as the cell counts returned to their initial levels.

DISCUSSION

It is now generally held that nitrogen mustard exerts its cytotoxic effects in a manner similar to x-ray irradiation.[14-16] It is seemingly more toxic to the rapidly proliferating cells and, in the bone marrow, produces an edema of the hematopoietic elements, cessation of mitotic activity, and ultimately hypoplasia or aplasia. The severity of myelosuppression is related to dosage and is reversible in the usual therapeutic range.[17] This marrow depression offers a reasonable anatomic explanation for the marked reduction of polymorphonuclear numbers in the peripheral blood some two weeks after drug administration. The rather typical changes in white cell counts described in this study attest to the fact that temporary myelosuppression was produced by the administered nitrogen mustard.

Knowledge of the biochemical changes that follow from the passage of radiation or radiomimetic agents through living organisms is still quite fragmentary. Excellent reviews of these investigations[18-19] are available, but they offer no unifying generalizations or conclusions regarding the effects of radiation on the enzymes of living tissue. Available studies on the effects of radiation on alkaline phosphatase are meager and yield conflicting results.[20-23] No support could be found for the possibility that the changes in leukocyte alkaline phosphatase described in the present study are due to a direct effect of nitrogen mustard on the enzyme. Further, in view of the time sequence described for the alkaline phosphatase changes and their correlation with the changes in polymorphonuclear cell numbers it seems highly unlikely that these changes

could be due to the direct effect of the radiomimetic agent. The evidence to date[24] suggests that the enzyme changes which follow radiation are more immediate than those described here. In vitro studies carried out in this laboratory, in which isolated leukocytes were incubated for one hour with varying concentrations of nitrogen mustard, showed no change in the alkaline phosphatase content of the cell.

An alternate explanation for the changes in the leukocyte alkaline phosphatase described, would involve an indirect effect of nitrogen mustard; namely, its nonspecific stress effect on adrenal cortical activity. We do not believe that the course of events described can be attributed to ACTH action on leukocyte alkaline phosphatase which is known to be more immediate in its effect, and more short-lived.[25]

The most likely explanation of the described data rests on the assumption that the neutrophil acquires increasing concentrations of alkaline phosphatase with physiologic and anatomic maturation. Cytochemical observations in support of this hypothesis have already been presented. There is also evidence that nondividing cells are capable of enzyme synthesis.[26] The neutrophil is anatomically a tissue cell in brief transit in the blood stream.[27] One may postulate that the polymorphonuclear cells are released from the active marrow in somewhat immature state, comparable to the reticulocyte, but, unlike the latter, leave the blood stream to reach their final stages of maturity and function in the tissues. Further, there is evidence that the total mass of leukocytes in the tissues is extremely large[27, 28] and that cells can shift from this reserve area to the blood stream. Whether all tissue leukocytes can re-enter the blood stream is still open to question.[29] There is general agreement, however, that there is a large intramedullary reserve of neutrophilic leukocytes which can supply cells rapidly to meet peripheral utilization. Thus, at any moment, the leukocytes in the blood constitute a mixed population of cells of recent and older descendents of the myeloid series.

As has been stated, nitrogen mustard acts most vigorously on the rapidly proliferating cells. The dampening of cell production should logically lead to a reduction in the younger cell population and leave essentially the mature and maturing populations which have escaped the cytotoxic effect of the drug. At the height, then, of the leukopenia that follows the nitrogen mustard administration, only mature cells should be present which would explain the rise in alkaline phosphatase levels described. This state of affairs should persist until such time as marrow activity returns and with the renewed production of leukocytes and the liberation of large numbers of "younger" cells, the leukocyte alkaline phosphatase should fall, as was observed. Further studies would be required in both clinical and induced leukopenic and hyperleukocytic states to test the above-postulated relationships of alkaline phosphatase activity and maturity.

Little of significance can be deduced from our data concerning the life of the white blood cell.

SUMMARY

Significant alterations in leukocyte alkaline phosphatase activity have been described following nitrogen mustard administration. These alterations, in general, moved in opposite direction to the changes in leukocyte numbers. The highest levels of enzyme activity were found at the nadir of cell depression. Possible mechanisms for these changes have been discussed. It is felt that the described findings are consistent with the hypothesis that alkaline phosphatase content may be a measure of polymorphonuclear cell maturity.

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THE RATE OF REMOVAL OF IRON FROM CULTURE MEDIUM BY HUMAN BONE MARROW SUSPENSION

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THE purpose of this report is (1) to describe an in vitro method that is suitable for a study of the rate of removal of iron from a culture medium by a suspension of human bone marrow cells and (2) to present the results that have been obtained with this technique.

METHODS

The suspension culture technique that was originally described by Osgood and Brownlee[6] and modified by Lajtha[1] was found suitable for these studies with but slight additional modification. The following procedure was used.

A 3 ml. sample of bone marrow was obtained in the usual manner from the iliac crest or the sternum. Films were made on glass slides for staining and counting and the remainder of the sample was injected into a sterile vaccine type vial containing 18 ml. of Heparin-Ringer's solution at 37° C. After the vial had been centrifuged for 10 minutes at 1,500 r.p.m., the supernatant was withdrawn and discarded. Five milliliters of Gey's solution were then injected into the vial and the suspension thoroughly mixed. Standard white cell pipettes and 0.1 N HCl were employed to count the nucleated cells present in this sample. Since it was found that a concentration of 4,000 to 7,000 nucleated cells per cubic millimeter in the final suspension gave satisfactory results, the initial concentration of nucleated cells in the sample was determined and the amount of Gey's solution necessary to produce a concentration of approximately 8,000 to 14,000 nucleated cells per cubic millimeter was added. The culture medium was prepared in each instance from compatible serum or from the serum of the patient whose marrow was being studied.

Lajtha[1] has reported that the serum concentration in the final culture may be varied from 20 to 95 per cent. The serum concentration in these studies varied from 20 to 50 per cent. Desired increases in the serum iron levels in the culture media were obtained by the addition of ferric chloride ($FeCl_3$) in 0.85 per cent saline. The concentration of the ferric chloride solution was such that it was unnecessary to add more than 0.1 ml. to a 3 ml. culture in order to obtain the desired iron level. In no instance was the iron binding capacity of the serum exceeded. The iron content after preparation of the culture medium, which consisted of that in the patient's serum and added iron, was determined by the method of Kitzes.[7] Radioactive iron (as $FeCl_3$) of high specific activity was then added to the prepared culture medium. The final concentration of Fe^{59} in the suspension culture was on the order of 0.007 to 0.01 μc.

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One and one-half milliliters of the cell suspension containing approximately 8,000 to 14,000 nucleated cells per cubic millimeter and 1.5 ml. of the prepared culture medium were then placed together in a 16 by 100 mm. tube with a vaccine-type stopper. The nucleated cell count of the final suspension was thus 4,000 to 7,000 nucleated cells per cubic millimeter.

The number of nucleated red blood cells present was determined from the ratio of total nucleated cells of all types to nucleated red blood cells. The range of nucleated red blood cells in these cultures was from $2 \times 10^6 \times 11 \times 10^6$ per 3 ml. culture. The final concentration of iron available to the individual red blood cell precursors depended both on the level of iron available in the culture and the concentration of nucleated red blood cells present in the culture. The use of a tube which fit both the roller tube rack and the well of the scintillation counter was advantageous as it allowed the entire operation to be carried out in the one tube.

The culture was thoroughly mixed and a final nucleated cell count obtained. After the tubes containing the suspension cultures had remained in a roller tube rack in an incubator at 37° C. for 24 hours, the activity of each tube was determined in a well-type scintillation counter. The tubes were then centrifuged at 1,500 r.p.m. for 10 minutes and the supernatant fluid removed and discarded. The cells were then washed 3 times with 0.85 per cent saline. Centrifugation was carried out and the supernatant fluid discarded between each washing. The activity in the supernatant fluid after the final washing was found to be essentially zero. This supernatant fluid was discarded and 3 ml. of distilled water was added to the infranatant cells and thoroughly mixed. The activity remaining in the final suspension was determined in the scintillation counter.

The absolute number of nucleated cells was calculated from the total nucleated cell count and a differential nucleated cell count made of the stained smear prepared at the time of the bone marrow suspension. As the calculations below are based on the total erythrocyte precursor count, the "average" cell referred to is, in reality, an average of the activity of the types of red cell precursors present in the marrow. These cells were predominantly polychromatophilic and orthochromic normoblasts.

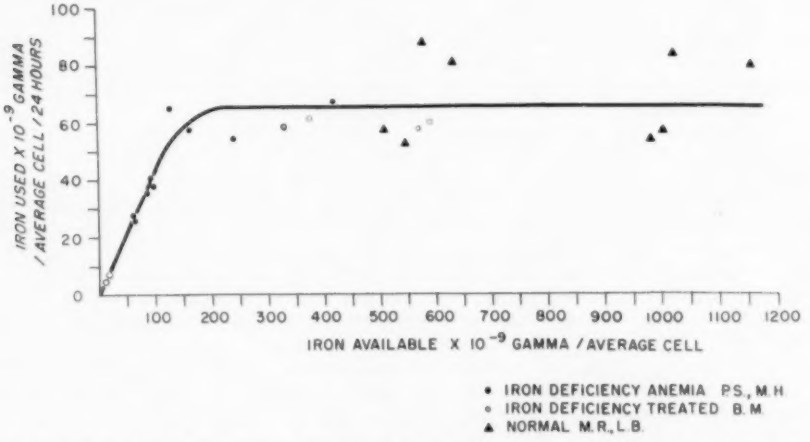
The amount of iron removed by the cell suspension from the culture medium in a 24-hour period may be calculated after determination of the specific activity. It should be pointed out that the activity, when determined at the end of 24 hours, is approximately equally divided between the cellular fraction which contains large numbers of nucleated red blood cell precursors and the supernatant hemolyzate. Tubes from which both cells and supernatant fluid had been removed showed no residual activity. The results indicate only the amount of iron removed from the culture medium in a 24-hour period and do not indicate the amount of hemoglobin produced during that period though the latter must represent some function of the total iron removed.

The 4 uremic patients from whom suspension cultures were obtained had blood urea levels above 150 mg. per cent and were anemic. The clinical diagnosis in 2 patients was chronic pyelonephritis and in the other 2 chronic glomerulonephritis. All died subsequently of their disease.

RESULTS

1. *The Effect of Decreasing the Concentration of Iron Available in the Culture.*—As the concentration of iron available to the developing red blood cell precursor was decreased below a level of 175 to 200×10^{-9} gamma per cell, the rate of removal of iron from the culture medium by the "average" cell decreased (Fig. 1). It will be noted, however, that marked increase in the concentration of iron above 175 to 200×10^{-9} gamma per cell did not result in a concomitant increase in the rate of removal of iron by the "average" erythrocyte precursor. The same data plotted in a different way to show the percentage removal of available iron by the "average" cell are presented in Fig. 2. As the

concentration of iron is decreased below a level of 175 to 200 $\times$ 10^{-9} gamma per nucleated red blood cell there is a concomitant increase in the percentage of iron removed from the culture by the developing erythrocyte. No difference was noted in the behavior of the marrow cells from patients with iron deficiency and those from normal individuals.


IRON USED X 10⁻⁹ GAMMA / AVERAGE CELL / 24 HOURS
0
20
40
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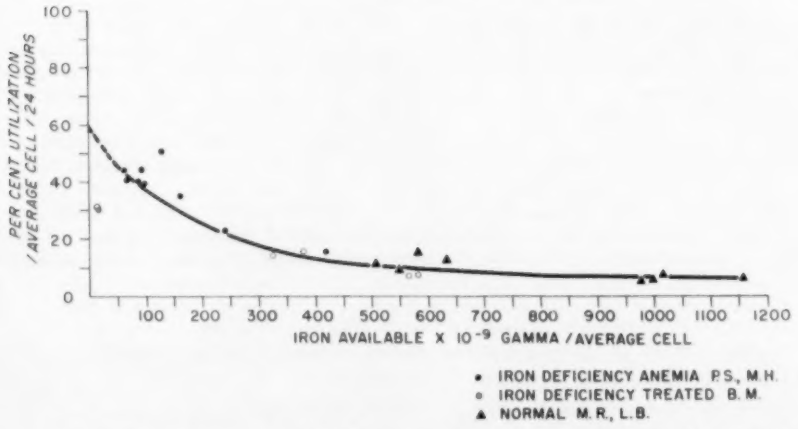
100 200 300 400 500 600 700 800 900 1000 1100 1200
IRON AVAILABLE X 10⁻⁹ GAMMA / AVERAGE CELL
• IRON DEFICIENCY ANEMIA P.S., M.H.
◦ IRON DEFICIENCY TREATED B.M.
▲ NORMAL M.R., L.B.

Fig. 1.—The relation of the level of iron in the culture medium to the amount removed in 24 hours. Each point represents a separate culture.


PER CENT UTILIZATION / AVERAGE CELL / 24 HOURS
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• IRON DEFICIENCY ANEMIA P.S., M.H.
◦ IRON DEFICIENCY TREATED B.M.
▲ NORMAL M.R., L.B.

Fig. 2.—The per cent of available iron utilized in 24 hours. The data from Fig. 1 are replotted in this manner to show the increasing proportion of available iron removed as the iron level is decreased.

2. *Failure of Peripheral Blood to Remove Iron From the Culture Medium.*—When peripheral blood was prepared in the same fashion as bone marrow aspirations, no removal of iron from the culture medium could be detected unless the

peripheral blood contained nucleated red blood cells or an increase in the number of reticulocytes. As other investigators have found previously[2] we found no detectable radioactivity in the stroma of peripheral blood cells.

3. *The Effects of Sodium Fluoride and of Low Temperature on the Removal of Iron From the Culture Medium.*—Sodium fluoride has been shown to interfere with the active transport of phosphate ions in the mature human erythrocyte through its inhibition of glycolysis.[8] A similar inhibition of iron transport was demonstrated in suspension culture of human bone marrow cells. Progressive increases in the molar concentration of sodium fluoride resulted in a stepwise decrease in the removal of iron from the respective cultures (Fig. 3). Continued removal of iron from the culture medium could not be stopped completely within the molar range of sodium fluoride used. The 0.009 and 0.014 M concentrations noted in Fig. 3 produced slight hemolysis and may represent slightly lower values than would have occurred in the absence of hemolysis. Higher concentrations of sodium fluoride caused marked hemolysis and consequent loss of iron to the discarded supernatant. Cultures maintained in the refrigerator at temperatures of 4° to 6° C. show essentially no uptake.

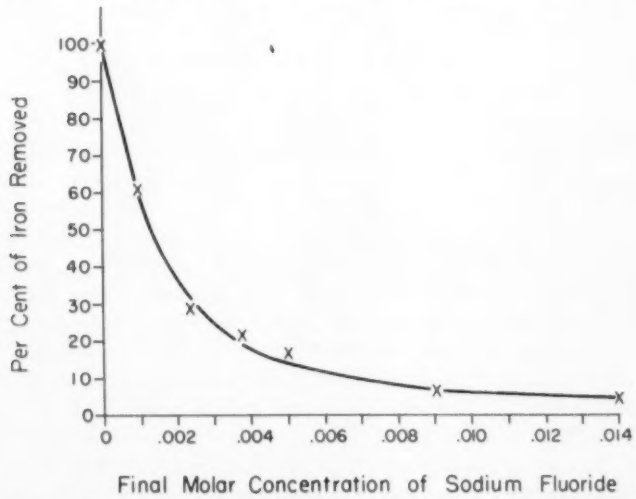

Fig. 3.—Influence of various concentrations of sodium fluoride on the percentage of iron removed from the culture medium in a 24-hour period. For purposes of this figure, the amount of iron removed in the absence of sodium fluoride was considered to be 100 per cent and the rate is, therefore, expressed as per cent of normal. The 7 cultures were obtained from the same subject. Values for NaF concentrations represent final concentration of the inhibitor in the culture.

4. *Rate of Removal of Iron From the Culture Medium by Cell Suspensions Obtained From Patients With Chronic Renal Insufficiency.*—Cell suspensions prepared from the marrow of patients with chronic renal insufficiency showed a consistently low rate of removal of iron from the culture media prepared from their own sera. The uptake of iron by the average erythrocyte precursor was

consistently below normal and values varied between 10×10^{-9} to 25×10^{-9} gamma per nucleated red blood cell per 24 hours in the asymptotic portion of the curve (Fig. 4).

Although the erythrocyte precursors in these cultures did remove a relatively normal percentage of iron from the culture medium when small amounts of iron were available, the rate of removal did not show the normal increase when the amount of available iron was increased.

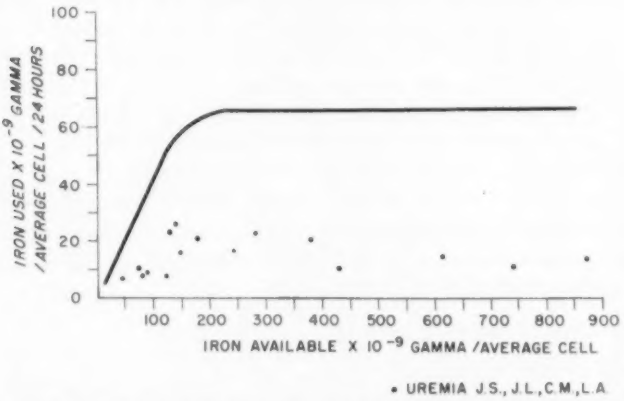

Fig. 4.—The relation of the level of iron in the culture media of sera from uremic patients to the amount removed by marrow cells from the same patients in a 24-hour period. The solid line representing the curve from Fig. 1 is drawn in for comparison.

DISCUSSION

The method described permits a study of the rate at which iron is removed from a culture medium by a suspension of human red blood cell precursors in vitro. By autoradiographic studies, Lajtha[2] found no evidence of uptake of iron by cells in marrow suspensions other than erythrocyte precursors. He concluded that the lack of appearance of Fe^{59} in any of the nonerythroid series indicated stability and slow turnover of the cytochrome iron in these cells. Studies of peripheral blood by Lajtha, and in our laboratory, have failed to reveal any nonspecific binding by the stroma of mature red or white blood cells.

In view of the remarkable increase in the percentage removal of iron from the culture medium as the available iron is decreased, the serum iron must be regarded as readily available to the developing erythrocyte under these culture conditions. In studying the uptake of iron by duck erythrocytes, Jensen and associates[9] noted a similar relationship between the quantity of iron available and the amount of iron removed from the culture medium.

The point at which the curve in Fig. 1 becomes asymptotic is at approximately 175×10^{-9} gamma of available iron per cell per 24 hours. This, then, represents the lowest level of iron concentration at which maximal removal of iron by the cells in these suspension cultures occurs. Since in the normal person 27 mg. of iron, with a range of from 21 to 37 mg.,[11] are transported in a 24-hour period by the plasma, approximately 1.5×10^{11} red blood cell precursors could

be exposed each 24 hours in vivo to that level of iron which in vitro has been found to be the lowest at which maximal removal will occur. It is interesting to note that as the life of the red blood cell is of the order of 120 days, approximately 2×10^{11} cells must be removed and replaced daily.

Recently, Markson, and Rennie[3] have reported that the proportion of polychromatophilic and orthochromic normoblasts was significantly less when human bone marrow suspensions were cultured in uremic serum than when cultured in normal serum. These data suggest either (1) there was a decrease in the number of maturing cells or (2) there was a decrease in the rate of individual cell maturation. While methods at present do not afford reliable data as to the numbers of cells involved, their results do indicate at least a decrease in the rate of maturation of the erythroid precursors. Erslev[5] has reported a decrease in erythropoietin in serum from rabbits made uremic, as well as an inability of the uremic rabbits to respond in the usual manner to erythropoietin. These observations agree with the present findings of a decreased rate of removal of iron from the culture medium by the marrow cells of uremic patients and the finding by Finch and associates[10] of a decreased iron utilization in vivo by patients with uremia. While a decrease in the number of developing erythrocytes or a decrease in their rate of maturation may explain our findings, another possibility remains. The results obtained with sodium fluoride and at low temperatures suggest that the incorporation of iron by the developing red blood cell is energy-dependent and that the energy for the reaction is, at least in part, obtained from glycolysis. Rees and co-workers[4] have reported that in vitro erythrocyte equilibration in uremic plasma reveals an inability of the cells to maintain normal phosphate and potassium balance. If patients with chronic renal disease do indeed have in their sera a substance or substances that will interfere with phosphate ester synthesis and potassium balance, the effect may be to reduce the energy potential of the cell and interfere with mechanisms by which the transfer of iron from the culture medium to the interior of the red blood cell precursor is carried out. Preliminary studies indicate that red blood cell precursors from uremic patients do not recover normal activity within a period of 24 hours when cultured in normal serum. On the other hand, the rate of iron removal by red blood cell precursors obtained from a normal subject and cultured in uremic serum was depressed. Further studies are under way to clarify the mechanism involved.

SUMMARY

1. A study of the rate of removal of iron from the culture medium by a suspension of human bone marrow cells in vitro is presented.

2. The iron in the culture medium is apparently readily accessible to the developing erythrocyte.

3. Sodium fluoride in concentrations below those producing hemolysis will depress but not abolish the removal of iron from the culture medium.

4. The rate of removal of iron from the culture medium by suspensions of cells obtained from patients with chronic renal disease and uremia is significantly below the expected rate.

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SERUM COMPLEMENT IN CONNECTIVE TISSUE DISORDERS

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SERUM complement has been found to vary from normal in several of the connective tissue disorders, including rheumatic fever,[1] rheumatoid arthritis,[2, 3] disseminated lupus erythematosus,[4] and dermatomyositis.[5] The present paper reports observations on serum complement levels in rheumatic fever, rheumatoid arthritis, disseminated lupus erythematosus, scleroderma, dermatomyositis, and periarteritis nodosa.

METHODS AND MATERIALS

Serum complement was determined by the procedure outlined by Fischel[1] after the method of Mayer and associates.[6, 7] Blood was obtained from patients at the New York Hospital, Bellevue Second Medical Division, the New York Postgraduate Hospital, the Massachusetts General Hospital, the Boston City Hospital, and the Hospital for Special Surgery. Bloods were allowed to clot at room temperature and after centrifugation at 2,500 r.p.m. for 3 minutes, 3 c.c. of serum was collected and stored at −70° C. No serum was allowed to remain at room temperature for more than 2 hours.

The amount of serum complement was determined by interpolation on the curve $x = \frac{y}{1-y}\left(\frac{1}{n}\right)$, where x = the amount of complement in 50 per cent hemolytic units and y = the degree of lysis. $\frac{1}{n}$ could not be assumed to remain constant at 0.2, as in ideal conditions, and slopes were calculated for each serum complement determination. This allowed an internal check on the consistency of the hemolytic curves of each specimen and also a check on the accuracy of dilution of each sample. No values were used with slopes outside an arbitrary range of 0.16 to 0.24, except in the case of some disseminated lupus erythematosus sera where large amounts of diluted serum were needed to demonstrate complement activity and slopes tended to be low.

Usually 2, and sometimes 3, tubes gave hemolysis between 10 per cent and 70 per cent and, therefore, could be accurately interpolated on the 50 per cent hemolytic curve. We chose to average the results of these 2 or 3 dilutions for our final value. Fischel[8] prefers to choose the value closest to 50 per cent lysis, but application of this criterion to our data did not change our individual results more than 1 to 2 units.

When an extremely low serum complement level was obtained, normal serum, diluted appropriately, was added to it in equal amounts to test for anticomplementary activity.

RESULTS

Serum complement determinations were performed upon 123 normal subjects, ages 18 to 60 and of both sexes (Fig. 1). Serum complement levels ranged

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from 35 to 54 U50 per cent* with a mean normal of 44 U50 per cent ± 1 S.D. of 4.7. This value for mean normalcy is higher than that previously reported by Fischel[1] as 37.7 U50 per cent ± 3.9, 1 S.D., but lower than Wedgewood and Janeway's normal[5] of 47.7 U50 per cent ± 13.3, 1 S.D. Several normal subjects were tested at varying intervals over a period of 4 months; serum complement levels did not vary more than 5 U50 per cent in these individuals. In addition, single serum specimens were tested at monthly intervals over a period of 6 months and were found to vary no more than 2 U50 per cent.

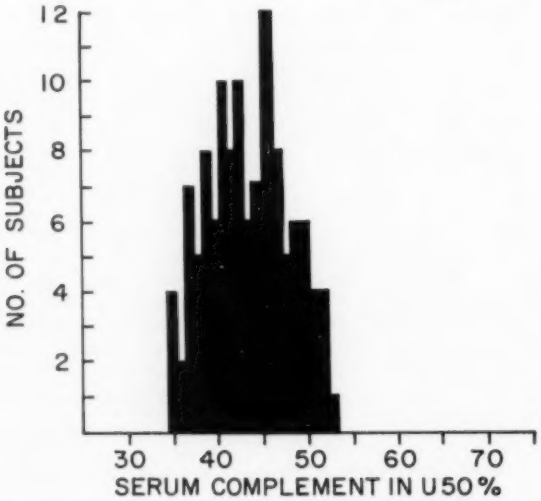

Fig. 1.—Serum complement levels in a group of 123 normal subjects. Mean complement value was 44 U50 per cent ± 4.7, 1 standard deviation.

Rheumatic Fever.—Serum complement levels were determined in 15 patients with acute rheumatic fever. The diagnosis of active rheumatic fever was determined according to the criteria of Jones.[9] Serum complement in 15 patients during the initial phases of rheumatic carditis ranged from 44 to 104 U50 per cent, with a mean value of 55 U50 per cent and median value of 57 U50 per cent. Standard deviations are not included because of the wide range of values. Serum complement levels of individual patients receiving adrenal corticosteroid therapy were noted to fall as rheumatic activity subsided. Occasionally, patients appearing clinically well were found to have a secondary rise in serum complement levels when corticosteroid therapy was discontinued.

Rheumatoid Arthritis.—Serum complement levels were studied in 48 patients with rheumatoid arthritis (Fig. 2). Patients were divided into 3 groups on the basis of clinical and laboratory findings.

Severe activity: (Stages III and IV by American Rheumatism Association Classification) This group showed elevations of temperature above 38° C.,

*U50 per cent refers to 50 per cent hemolytic units of complement per milliliter of serum.

multiple active joint involvement with synovitis and effusions, and sedimentation rates above 40 mm. per hour (Wintrobe). They were confined to bed because of joint pain and fixation.

Moderate activity: (Stage II, A. R. A.) These patients had temperatures of 37 to 38° C., one or more joints involved in active synovitis, sedimentation rates between 20 and 60 mm. per hour (Wintrobe), and were ambulatory.

Mild activity: (Stage I, A. R. A.) This group had no fever, one and occasionally two joints involved with capsular thickening or minimal effusion (but few signs of active synovitis), and sedimentation rates of 15 to 40 mm. per hour (Wintrobe). They also were ambulatory patients.

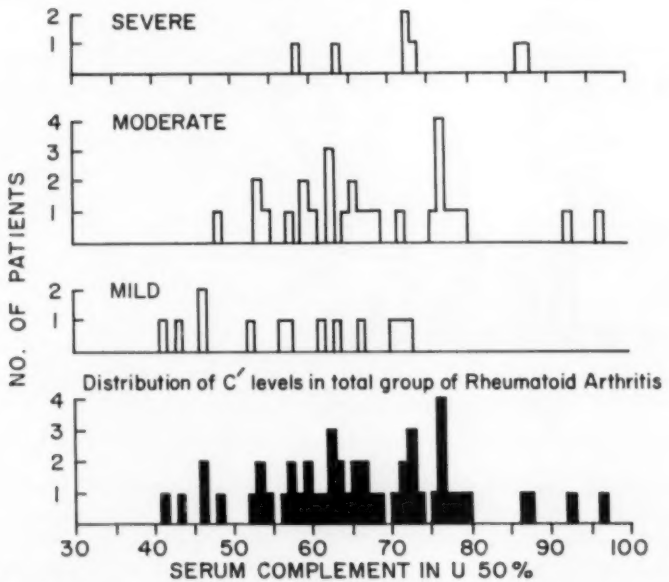

Fig. 2.—Range of serum complement levels in 48 patients with rheumatoid arthritis.

Serum complement levels in the entire group ranged from 42 to 97 U50 per cent ± 12, 1 S.D. The range of serum complement levels in 7 patients classified as *severe* was 59 to 88 U50 per cent, with a median value of 73 U50 per cent. Twenty-eight patients with *moderate* rheumatoid activity had serum complement values from 49 to 97 U50 per cent with a mean of 69 U50 per cent and median of 67 U50 per cent. Thirteen patients with *mild* rheumatoid activity were found to have a range of serum complement values from 42 to 73 U50 per cent with a mean and median of 58 U50 per cent (Figs. 2 and 6). A suggestive relationship between the degree of activity of the rheumatoid process and the elevation of serum complement is noted in comparing the 3 groups, but the only significant difference is found in comparing the mild category with the moderate or severe groups ($P<0.01$).

In two patients treated with ACTH and adrenal corticosteroid, serum complement levels were noted to decrease after the institution of treatment. Four of 8 patients who responded clinically to gold therapy continued to have elevated serum complement levels, while 4 showed decreasing levels with clinical improvement.

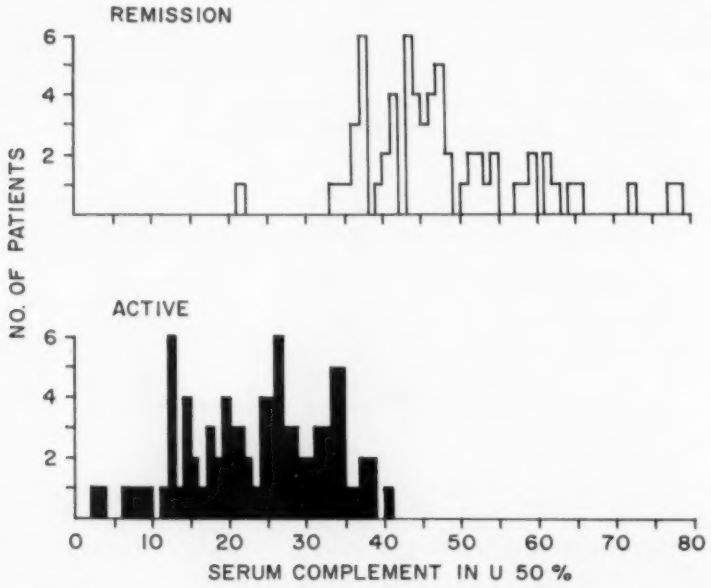

Fig. 3.—Serum complement levels in 58 patients with disseminated lupus erythematosus.

Disseminated Lupus Erythematosus.—One hundred and fifty-seven determinations of serum complement were performed in a series of 58 patients with disseminated lupus erythematosus (Fig. 3). All patients studied showed positive lupus erythematosus preparations at some time in their course. Patients were divided into two groups—one representing the *active* disseminated phase, the other consisting of patients considered to be in remission.

Active disseminated lupus erythematosus: Patients were placed in the *active* group on the basis of clinical and laboratory evidence, i.e., skin rash, arthritis, temperature of 38° C. or above, purpura, serositis, elevation of sedimentation rate above 20 mm. per hour (Wintrobe), anemia, and evidence of active renal involvement as manifested by albuminuria and casts, red cells, and white cells in urinary sediment.

Remission—disseminated lupus erythematosus: Patients classified as being in remission were either those undergoing spontaneous clinical remission or those who were well controlled on adrenal corticosteroid and salicylate therapy.

In the *active* group which comprised 44 patients, the mean serum complement level was found to be 24 U50 per cent ± 8.9, 1 S.D. This is significantly

below the normal mean of 44 U50 per cent ($P<0.01$). Determinations on the sera of 34 patients during remission gave a mean value of 53 U50 per cent ± 11.6, 1 S.D. Slow increases in serum complement levels were noted in association with a transition from the active phase to one of remission, whether spontaneous or drug-induced. Patients with active disseminated lupus who failed to show remission during corticosteroid therapy continued to have subnormal complement levels. Of particular interest are 3 patients with rheumatoid arthritis who presented positive Hargreaves' preparations at some time in their course in association with elevated sheep cell agglutination titers. Sera tested at the time lupus erythematosus cells were demonstrable showed complement values ranging from 47 to 76 U50 per cent in 6 determinations. These levels were in the range of the 48 patients tested with rheumatoid arthritis, and far above the range noted for active disseminated lupus.

Three patients were studied who underwent splenectomy for thrombocytopenia associated with disseminated lupus erythematosus. All 3 had a rise in serum complement level to normal and supranormal levels with good platelet response after splenectomy. However, two of these patients later showed low serum complement values as their disease became active with other visceral involvement.

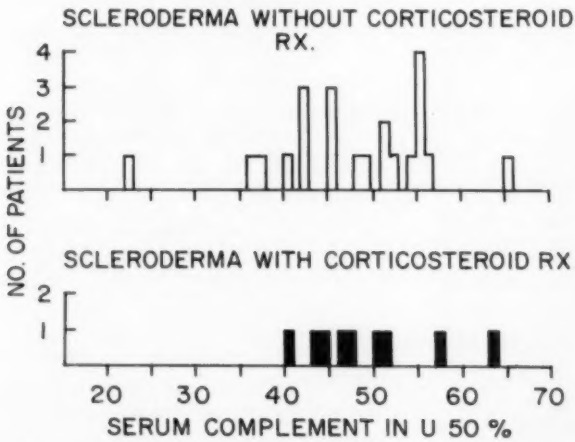

Fig. 4.—Serum complement levels in 30 patients with scleroderma.

Scleroderma.—Thirty patients with well-documented scleroderma were studied (Fig. 4). Those receiving maintenance adrenal corticosteroid therapy were compared with those not under such therapy. In 9 corticosteroid-treated patients, the mean serum complement value was 50 U50 per cent with a range of 41 to 64 U50 per cent, whereas in the 22 patients who were not receiving corticosteroids, the mean was 48 U50 per cent ± 8.8, S.D., with a range of 23 to 66 U50 per cent (Fig. 4). No significant difference was noted between these two groups and normal individuals, or between the two groups themselves.

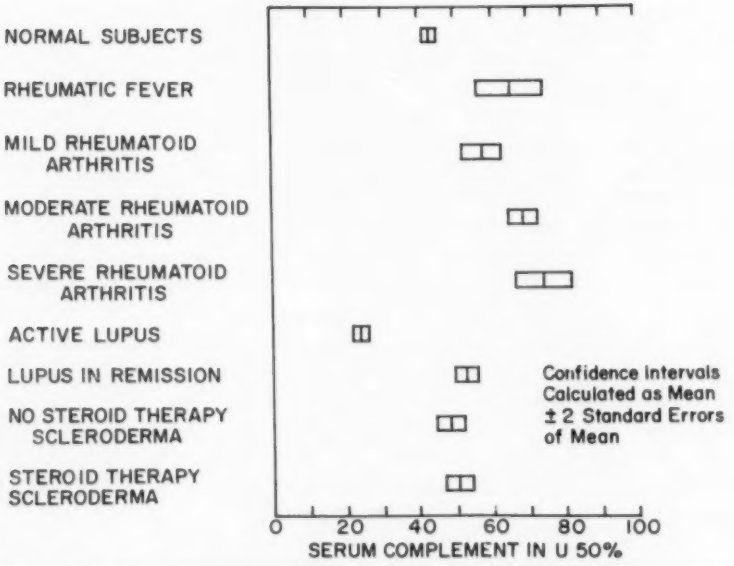

Fig. 5.—Confidence intervals and means of groups of patients with connective tissue diseases. A significant difference is noted between means of two groups when there is no overlap in confidence interval.

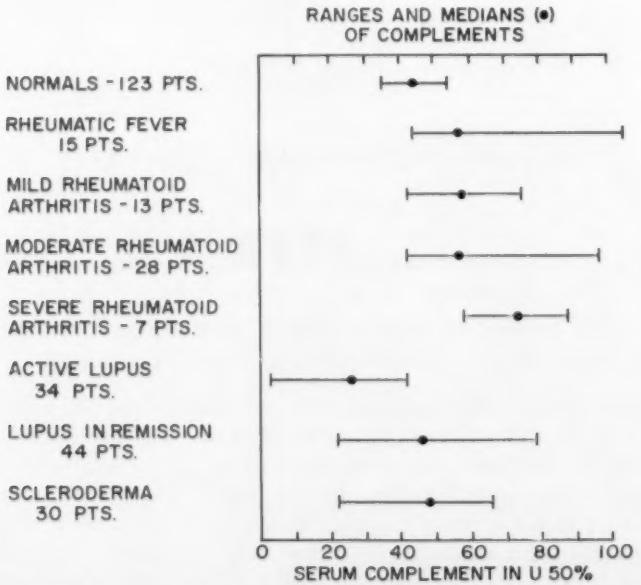
RANGES AND MEDIANS (•) OF COMPLEMENTS
NORMALS - 123 PTS.
RHEUMATIC FEVER 15 PTS.
MILD RHEUMATOID ARTHRITIS - 13 PTS.
MODERATE RHEUMATOID ARTHRITIS - 28 PTS.
SEVERE RHEUMATOID ARTHRITIS - 7 PTS.
ACTIVE LUPUS 34 PTS.
LUPUS IN REMISSION 44 PTS.
SCLERODERMA 30 PTS.
0 20 40 60 80 100
SERUM COMPLEMENT IN U 50%

Fig. 6.—Ranges and median values of serum complement in groups of connective tissue diseases studied.

Periarteritis Nodosa and Dermatomyositis.—Five patients with periarteritis nodosa were studied. In each instance, the diagnosis was proved by biopsy or at autopsy. Serum complement values ranged between 49 and 65 U50 per cent. This series was too small to attempt a correlation of complement levels and disease activity.

In 10 determinations on 7 patients with dermatomyositis, serum complement values ranging from 96 to 36 U50 per cent were found. The value of 96 U50 per cent was present in one patient during an acute exacerbation of her dermatomyositis. The serum complement level subsequently fell to 35 U50 per cent during corticosteroid-induced remission. At autopsy, 1½ years later, widespread ovarian carcinoma was found in addition to chronic dermatomyositis.

Comparison of General Groups.—Confidence intervals were determined by plotting the mean and two standard errors of the mean in each group of patients studied. Significant differences in means were apparent when no overlap in confidence intervals occurred (Fig. 5).[10]

The range and median in each group are presented in Fig. 6.

DISCUSSION

Serum complement levels were found to be elevated in acute rheumatic fever and rheumatoid arthritis. Active disseminated lupus erythematosus was found to be associated with low levels of serum complement; when a remission of disseminated lupus erythematosus was achieved, the serum complement levels rose to normal or supranormal levels. Differences in these groups were shown to be statistically significant using the confidence interval technique.[10] There was a significant difference between the mean serum complement levels found in active disseminated lupus erythematosus and the mean serum complement level obtained in rheumatic fever, rheumatoid arthritis, scleroderma, and disseminated lupus erythematosus in remission.

There has been some disparity among earlier investigators as to changes in serum complement levels in acute rheumatic fever. Initially low levels in the acute phase of rheumatic fever were reported by Veil and Buccholz,[11] Rachmilewitz and Silberstein,[2] and Coburn.[12] Goldberg and DeGara[13] found normal to slightly decreased serum complement levels in their cases of active carditis. Our data in rheumatic fever are in agreement with the more recently published work of Fischel[1] and Wedgewood and Janeway[5] who noted elevations of serum complement with rheumatic activity. In addition, our results are in accordance with the data published concerning rheumatoid arthritis by Vaughan and associates[3] and Wedgewood and Janeway[5] reporting complement elevations with rheumatoid activity. Low serum complement values in disseminated lupus erythematosus have been reported previously by Elliott and Mathieson[4] and Wedgewood and Janeway.[5]

Little is known concerning the mechanism of serum complement increase or decrease in the connective tissue disorders. If complement were uniformly increased, a nonspecific reaction to the various inflammatory processes might be postulated. This in fact may be the case, since serum complement is involved

in numerous immunologically detectable responses to inflammation invoked by infection. Rises in serum complement levels have also been noted in association with the inflammatory response to myocardial infarction[14] and malignancy.[15]

One of the most widely held explanations for serum complement depression in any disorder is the hypothesis that antigen-antibody reactions fix complement both in vitro[16] and in vivo.[17] Diminution of serum complement has been reported in instances of serum sickness and penicillin reactions.[4]

Other possibilities exist to account for decreased serum complement in such diseases as disseminated lupus erythematosus. Decreased ability to produce serum complement could be postulated. This does not seem likely; several of our patients with the superimposed stress of infection or trauma showed an ability to raise their serum complement from previous low levels.

Apparent decreases in serum complement may be due to the presence of anticomplementary substances in the sera. In our patients, sera were spot-tested for anticomplementary activity, and none could be demonstrated. Anti-complementary activity has been attributed to increased gamma globulin components by Vaughan, Bayles, and Favour,[18] who studied simultaneous variations in serum complement and gamma globulin, noting an inverse relationship. However, these authors did not feel that such a simple explanation for low serum complement levels in disseminated lupus erythematosus was warranted because elevations of gamma globulins were not associated with low complement levels in other disease states such as rheumatoid arthritis. In our patients, 15 sera with very low serum complement levels were subjected to electrophoresis in order to estimate gamma globulin content. Eight of these sera showed moderate increase in gamma globulin content, but in no way as great as the increases in gamma globulin in 15 sera from patients with rheumatoid arthritis who had *elevated* serum complement levels.

A correlation appeared to exist between very low serum complement levels in disseminated lupus erythematosus and extensive skin or renal involvement. This may reflect merely the activity of the disease process itself and does not seem to be the specific criterion for serum complement depression. It is of interest to note that acute glomerulonephritis and the nephrotic syndrome have been reported to be associated with marked depressions in serum complement levels.[19-21]

SUMMARY

1. Serum complement determinations using the 50 per cent hemolytic method were carried out on 123 normal subjects and a series of patients with various connective tissue disorders.

2. Normal levels of serum complement were found to vary about a mean of 44 50 per cent hemolytic units ± 4.7, 1 standard deviation.

3. Elevated serum complement levels were found in patients with rheumatoid arthritis and acute rheumatic fever.

4. Low serum complement levels were present in patients with active disseminated lupus erythematosus; these low levels returned to normal as patients went into spontaneous or drug-induced remission.

5. Patients with scleroderma did not show serum complement levels significantly different from those of normal subjects.

6. Patients with active dermatomyositis and periarteritis nodosa exhibited elevations of serum complement.

We are indebted to Dr. I. D. J. Bross for help in the statistical methods used in this paper. Dr. A. W. Duryee of the New York Postgraduate Hospital furnished us with blood from many of the scleroderma patients studied. Dr. H. Barnett, Professor of Pediatrics, Albert Einstein College of Medicine, provided valuable suggestions in the course of this work.

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THE MECHANISM OF ACTION OF PREPARATIONS OF THROMBIN ON HEMOLYSIS OF RED CELLS FROM PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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THE addition of commercially available preparations of bovine thrombin to normal human serum in vitro results in marked augmentation of the hemolytic activity of the serum for red cells from patients with paroxysmal nocturnal hemoglobinuria (PNH). It has been suggested that this effect may result from decreasing the activity of an inhibitor of PNH hemolysis in normal serum.[1]

In the present studies, thrombin preparations which were altered so that they were almost devoid of clotting activity had the same effect on PNH hemolysis as preparations with active clotting properties. All the preparations tested agglutinated both normal and PNH cells. Since PNH cells are markedly susceptible to hemolysis by agglutinating antibodies, these observations suggest that antibodies to red cells present in thrombin preparations are responsible for the effect of thrombin on the hemolysis of PNH erythrocytes.

MATERIALS AND METHODS

Serum.—Fresh serum of the same or compatible ABO blood group was obtained from healthy donors by defibrination of venous blood with glass beads. Serum was obtained within 24 hours of use and stored at 4° C.

Erythrocytes.—Red cells were obtained from two patients with PNH, one of blood group A (Case 1) and one of blood group O (Case 2). Red cells were stored for not more than 7 days in modified Alsever's solution (41.0 Gm. glucose, 16.0 Gm. sodium citrate, 8.2 Gm. sodium chloride, adjusted to pH 6.1 with 10 per cent citric acid, and diluted to 2,000 ml.) at 4° C. At the time of use, erythrocytes were washed 3 times in isotonic sodium chloride, and finally suspended to a concentration of approximately 35 per cent in isotonic sodium chloride.

Hemolytic System.—Normal human serum was acidified to pH 6.8 with N/7 HCl, and one-tenth volume of 2 times osmotic imidazole buffer at pH 6.8 was added as previously described.[2] The total amount of diluent added to serum was always kept below 50 per cent of the original serum volume because further dilution resulted in marked decrease in hemolysis of PNH red cells. Ten volumes of serum mixture were added to one volume of cell suspension in the final test system. Incubation was at 37° C. for 30 minutes.

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The degree of hemolysis was determined by measuring colorimetrically the hemoglobin released into the serum and expressing the pigment released as the percentage of test cells hemolyzed. Hemolysis at pH 8 (± 0.2 pH unit) was tested using serum from defibrinated blood without any adjustment of the pH, but with addition of isotonic sodium chloride to make the dilution the same as that used at pH 6.8.

Agglutination of cells was determined by macroscopic inspection after centrifugation at 1,800 g for 2 minutes, and was scored from 0 to 4+.

Thrombin.—Solutions of bovine thrombin were made in isotonic sodium chloride from preparations that were available commercially. Three separate samples of Parke-Davis Thrombin Topical and one sample of Upjohn Thrombin were tested. Each of these samples gave reproducible results in the PNH hemolytic system. Thrombin preparations were diluted so that the desired amount was contained in one-tenth the volume of serum used. Although 50 units of thrombic activity per milliliter of serum have been used in the thrombin test as described by Crosby,[1] as little as 10 units of thrombic activity per milliliter had an effect on PNH hemolysis equal to the larger amount. Excessive amounts of thrombin are anticomplementary and inhibit PNH hemolysis. In the tests reported here thrombin solutions were diluted so that 0.1 ml. contained either 10 or 50 units of thrombic activity for clotting. Solutions of thrombin heated at 56° C. for 30 minutes contained less than 0.3 per cent of the activity of the original material in clotting a solution of fibrinogen. Solutions containing 100 units per milliliter of thrombic activity for clotting were adsorbed with an equal volume of packed normal human erythrocytes for 60 minutes at 4° C. with frequent mixing. A single adsorption resulted in loss of 0 to 15 per cent of thrombic activity.

A preparation of human thrombin, kindly supplied by Dr. E. B. McLean of Cutter Laboratories, was treated and used in the same manner as the bovine thrombin preparations.

The thrombic activity of the various preparations was determined by measuring the time of the initial appearance of a fibrin clot when 0.2 ml. of a solution containing 400 mg. per 100 ml. of human fibrinogen (Cutter) was mixed at 37° C. with 0.2 ml. of the preparation to be tested.[10] Dilutions of the preparation to be tested were made in 0.15 M barbital buffer containing 0.00015 M $CaCl_2$ at pH 7.4. Comparisons of thrombic activity were based on dilutions of the thrombin preparations that resulted in clotting in 20 to 30 seconds under the above conditions. Activity of the test preparations was derived from a standard curve of activity plotted each day, using dilutions of a thrombin preparation of known activity.

Properdin.—Measurement of properdin in thrombin preparations was performed according to the method of Pillemer and associates[3] on dilute solutions of thrombin since high concentrations of thrombin interfere with measurement of properdin. No properdin activity was demonstrated in any of the thrombin preparations. This is in contrast to previously reported[8] observations in which properdin activity was demonstrated in some preparations of thrombin.

RESULTS

Bovine Thrombin.—

1. Effect of preparations of bovine thrombin on hemolysis and agglutination of PNH and normal erythrocytes: At pH 6.8 normal human serum hemolyzed 28 per cent of the erythrocytes from Case 2 (Table I), and there was no agglutination. Addition of 50 units of a bovine thrombin preparation per milliliter to this serum resulted in an increase in the hemolysis to 44 per cent. Marked agglutination was observed when the bovine thrombin was added. Normal cells were agglutinated to similar degree, but were not hemolyzed. Group A and group O erythrocytes were affected similarly.

Although hemolysis of PNH cells by normal serum is minimal or absent at pH 8, hemolysis of PNH cells by isoantibodies occurs regularly at pH 8. Addition of bovine thrombin to serum at pH 8 caused marked hemolysis of PNH cells, agglutination of PNH and normal cells, but no hemolysis of normal cells.

TABLE I. EFFECT OF A PREPARATION OF BOVINE THROMBIN ON PNH AND NORMAL ERYTHROCYTES

	PNH CELLS (CASE 2)		NORMAL CELLS	
	HEMOLYSIS (%)	AGGLUTINATION	HEMOLYSIS (%)	AGGLUTINATION
pH 6.8				
Serum	28	0	0	0
Serum + thrombin (50 units per ml.)	44	3+	0	3+
pH 8.0				
Serum	0	0	0	0
Serum + thrombin (50 units per ml.)	38	3+	0	3+

TABLE II. COMPARISON OF EFFECT OF ANTI-A ISOANTIBODY AND A PREPARATION OF BOVINE THROMBIN AT pH 8 ON GROUP A PNH AND NORMAL RED CELLS

	GROUP A PNH CELLS (CASE 1)		GROUP A NORMAL CELLS	
	HEMOLYSIS (%)	AGGLUTINATION	HEMOLYSIS (%)	AGGLUTINATION
pH 8.0				
Serum	0	0	0	0
Serum + anti-A	3+	4+	tr	4+
Serum + thrombin	3+	3+	0	3+

TABLE III. GREATEST DILUTION OF A PREPARATION OF BOVINE THROMBIN SOLUTION AND ANTI-A ISOANTIBODY IN NORMAL HOMOLOGOUS SERUM AFFECTING GROUP A PNH AND NORMAL RED CELLS AT pH 8

	DILUTION OF REAGENT IN GROUP A SERUM			
	GROUP A PNH CELLS (CASE 1)		GROUP A NORMAL CELLS	
	HEMOLYSIS	AGGLUTINATION	HEMOLYSIS	AGGLUTINATION
pH 8.0				
Thrombin (initial concentration 50 units per ml.)	1/32	1/32	—*	1/32
Anti-A	1/128	1/128	1/2	> 1/128

*No hemolysis of normal cells occurred when 50 units of thrombic activity was added to serum.

2. Comparison of the effect of preparations of bovine thrombin and isoantibody on hemolysis of PNH cells: As seen in Table II the effect of preparations of bovine thrombin and anti-A isoantibody (contained in group O serum) on the hemolysis of type A PNH cells at pH 8 was qualitatively similar. Addition of either the thrombin preparation or anti-A resulted in marked hemolysis of PNH cells and agglutination of both normal and PNH cells. Serial dilution of a preparation of bovine thrombin in normal compatible serum resulted in disappearance of the agglutinating and hemolytic activities for PNH cells at approximately the same degree of dilution, 1 to 32 (Table III). Serial dilution

of anti-A isoantibody from normal group O serum gave similar results. Thus, the hemolytic property for PNH cells of a preparation of bovine thrombin and of a known antibody disappeared at the same degree of dilution as the agglutinating property.

3. Inactivation of bovine thrombin by heat: The effect of a solution of bovine thrombin on the hemolysis and agglutination of PNH cells at pH 6.8 and 8.0 was unaltered by heating the thrombin at 56° C. for 30 minutes. This occurred even though the residual thrombic activity for clotting was reduced to less than 0.3 per cent of the unheated control (Table IV).

TABLE IV. EFFECT OF HEATING BOVINE THROMBIN PREPARATIONS

	PNH CELLS (CASE 1)		THROMBIC ACTIVITY (PER CENT OF ORIGINAL)
	HEMOLYSIS (%)	AGGLUTINATION	
pH 6.8			
Serum	42	0	—
Serum + thrombin (50 units per ml.)	85	3+	100
Serum + heated thrombin	84	3+	< 0.3

4. Adsorption of bovine thrombin with human erythrocytes: As indicated in Table III, the preparations of bovine thrombin used had a marked effect on hemolysis of PNH cells at a much lower concentration than 50 units of thrombic activity per milliliter. Addition of 10 units per milliliter of serum (0.1 ml. of a solution containing 100 units of thrombic activity per milliliter) resulted in marked increase in PNH hemolysis. When a preparation of bovine thrombin containing 100 units per milliliter was adsorbed one time with an equal volume of packed human erythrocytes at 4° C. for 60 minutes, there was a marked decrease in the property of this partially adsorbed preparation to cause agglutination and to increase hemolysis of PNH erythrocytes when it was added to serum (Table V). There was only minimal loss of thrombic activity for clotting following adsorption compared to the large decrease in activity for hemolysis and agglutination.

TABLE V. COMPARISON OF ACTIVITY OF BOVINE THROMBIN PREPARATIONS BEFORE AND AFTER ADSORPTION BY NORMAL HUMAN RED CELLS

	PNH CELLS (CASE 2)				THROMBIC ACTIVITY (PER CENT ORIGINAL)
	pH 6.8		pH 8		
	HEMOLYSIS (%)	AGGLUTINATION	HEMOLYSIS (%)	AGGLUTINATION	
Serum	21	0	0	0	—
Serum + thrombin (10 units per ml.)	43	3+	17	2+	100
Serum + adsorbed thrombin (10 units per ml.)	27	±	0	±	95

Preparation of Human Thrombin.—A preparation of thrombin made from human plasma (Cutter) was observed to cause augmentation of hemolysis of group A, but not of group O PNH erythrocytes. Agglutination of group A cells

from normal persons and from the PNH patient was observed, but no agglutination of group O cells occurred. The effect on group A cells was markedly reduced by addition of A substance to the human thrombin preparation, or by adsorption of human thrombin with normal group A red cells but not by group O cells. Thrombic activity was not altered by these procedures. The human thrombin preparations were difficult to standardize in these systems because they had marked anticomplementary properties which were altered in variable fashion by heating and other manipulations. Therefore, it was not possible to determine conclusively that the effect of human thrombin was due only to its content of anti-A isoantibody.

DISCUSSION

Normal serum causes marked hemolysis of PNH erythrocytes at pH 6.8, but has minimal or no effect at pH 8.0. This hemolytic activity requires the properdin system.[6] No agglutination occurs and antibodies have not been demonstrated to be essential or involved in this hemolytic reaction. However, PNH cells are hemolyzed by certain antibodies to red cells in amounts sufficient to cause only agglutination of normal cells.[4, 5] This reaction requires complement, occurs at pH 8.0 as well as pH 6.8, and does not require properdin. There is no demonstrated relationship between the increased susceptibility of PNH cells to hemolysis by antibodies and the defect causing hemolysis of PNH cells by normal serum in the properdin system.

Preparations containing bovine thrombin added to compatible human serum caused a marked increase in the hemolysis of PNH cells at pH 6.8 and caused considerable hemolysis of PNH cells at pH 8. Those preparations also produced agglutination of normal and PNH cells of group A and group O, but did not cause hemolysis of normal cells. When thrombin was diluted serially in normal serum the hemolytic effect and agglutinating property for PNH cells disappeared at the same degree of dilution. Human isoantibodies affected PNH cells similarly. Because of the marked difference in susceptibility to the action of antibodies between PNH and normal cells, it appears that the effect of bovine thrombin was due to amounts of heterophil antibody sufficient to result in lysis of PNH cells but insufficient to hemolyze normal cells. Large concentrations of preparations of thrombin fail to result in hemolysis because they are anticomplementary, even though they cause marked agglutination. Heating at 56° C. for 30 minutes almost completely destroys thrombic activity for clotting, but does not inactivate heterophil or isoantibodies. Heating had no effect on the hemolytic activity of bovine thrombin preparations on PNH cells. Adsorption of preparations of bovine thrombin with human red cells resulted in parallel and marked decrease in hemolytic and agglutinating activities for PNH cells, but there was minimal loss of thrombic activity for clotting.

Observations on a preparation of human thrombin offered some confirming evidence. This preparation caused agglutination and augmented hemolysis of group A but not of group O PNH cells. This activity was diminished by

adsorption of the preparation of thrombin with normal group A cells or by addition of A substance, with slight or no decrease in thrombic activity. These data suggest that the naturally occurring anti-A isoantibody, present in pooled normal plasma from which thrombin is made, may have been responsible for the hemolytic effect of human thrombin on PNH hemolysis. Conclusive proof was not possible because of the interfering anticomplementary properties of human thrombin.

These observations distinguish between the effect of preparations of bovine or human thrombin on PNH cells and the thrombic activity as related to the clotting mechanism. Thus, the effects on PNH cells were markedly reduced by adsorption or neutralization of antibody, with little or no effect on thrombic activity. Virtually complete inactivation of thrombic activity occurred on heating, although there was no alteration of the hemolytic effect on PNH cells.

These findings fail to support the concept that thrombic activity per se plays a part in the hemolysis of PNH red cells. It seems more likely that preparations of bovine thrombin contain a heterophil antibody and that preparations of human thrombin contain an isoantibody that increases hemolysis of PNH red cells. It has been variously proposed that AC globulin, thrombin, or unknown factors are responsible for PNH lysis or act to remove possible inhibitors of the PNH reaction from serum.[7, 8] These conclusions were based in large part on the effect of preparations of thrombin or of AC globulin that were observed to increase PNH hemolysis. Present studies indicate that all, or most, of the effects on PNH hemolysis of thrombin preparations available commercially are unrelated to their activity for clotting, but are due to their content of antibodies to red cells or to antibody-like substances.

Although thrombin preparations tested and reported earlier from this laboratory contained properdin activity,[6] subsequent preparations have not contained measurable properdin activity. Others have indicated lack of properdin activity in thrombin preparations.[9] It cannot be determined whether the effect of the earlier reported preparations was due to properdin or to antibody to red cells, for they were not tested for agglutinating activity. It is unlikely, however, that the effect of bovine thrombin is due to properdin as suggested earlier.[6]

It appears warranted to conclude that the addition of bovine thrombin to human serum serves as a diagnostic aid in paroxysmal nocturnal hemoglobinuria because it adds a heterophil antibody which hemolyzes PNH cells but does not hemolyze normal red cells. This does not detract from its diagnostic usefulness, although similar results could be obtained using sera containing isoantibodies, or animal sera containing antihuman red cell antibodies.

SUMMARY

1. Four preparations of bovine thrombin agglutinated human erythrocytes. One preparation of human thrombin agglutinated group A but not group O erythrocytes.

2. The augmentation of hemolysis of PNH cells in normal serum by preparations of bovine and human thrombin paralleled the agglutinating activity for human red cells. Normal red cells were not hemolyzed.

3. Heating of preparations containing bovine thrombin reduced thrombic activity but did not decrease the agglutinating or hemolytic activities for PNH cells. Adsorption with human cells resulted in decrease in agglutinating and hemolytic properties without significant loss in thrombic activity.

4. The preparation of human thrombin caused augmentation of hemolysis of PNH cells of group A but not those of group O. This property was diminished, as was the agglutinating effect, after adsorption with group A cells or the addition of group A substance. These procedures had little effect on thrombic activity.

5. These data suggest that the effect of preparations of bovine and human thrombin on hemolysis of PNH erythrocytes is due to their content of heterophil or isoantibody or to antibody-like substances and not to thrombic activity for clotting.

I wish to thank Dr. O. D. Ratnoff for his assistance in performing the assays of thrombic activity.

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LABORATORY METHODS

AN ANALYSIS OF TECHNICAL ERRORS IN RADIOALBUMIN BLOOD VOLUME METHODS AND PRESENTATION OF A MODIFIED METHOD

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DURING the course of an investigation involving blood flow measurements it became necessary to evaluate and minimize the technical errors that occur in blood volume determination using I^{131}-labeled human serum albumin. In our hands, the largest single source of error resulted from the use of a calibrated syringe to deliver a known amount of radioalbumin. This error may be eliminated provided a means is available to deliver identical amounts of the labeled material to the patient and to a known volume. The purpose of this paper is to report: (1) the results of a systematic investigation of errors involved in blood volume measurement with radioalbumin; (2) a modified method for measuring blood volume which eliminates the use of a calibrated syringe; and (3) values obtained with this method for blood volume in a group of normal men living at an altitude of 1 mile above sea level.

METHODS AND MATERIALS

Theory.—

Blood volume: According to simple isotope dilution theory, an unknown volume (V_u) may be calculated if the activity (A_s) and volume (V_s) of the injected isotopic material and the activity (A_u) of the diluted isotopic material are known. This may be expressed as follows:

$$V_u = V_s \left[\frac{A_s}{A_u} - 1 \right] \qquad (1)$$

It should be noted that any error occurring in the amount injected (V_s) manifests itself as an error in the unknown volume (V_u).

It is also possible to determine the unknown volume (V_u) without knowing the volume of the injected material (V_s) provided reproducible volumes of the injected material

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can be delivered; i.e., identical volumes delivered both to the patient and to a known volume. The activity (A_u) of the isotope in the unknown volume (V_u) is given by:

$$A_u = \frac{\mu}{V_u + V} \tag{2}$$

where μ is the number of microcuries contained in volume V. If an identical volume is delivered into a known volume (V_s), the activity (A_s) is given by:

$$A_s = \frac{\mu}{V_s} \tag{3}$$

and V_u is obtained by combining equations 1 and 2.

$$V_u = V_s \frac{A_s}{A_u} - V \tag{4}$$

Note that V does not appear as a factor in equation 4 and thus if V_s is chosen very large with respect to V, the latter is negligible. As in any dilution technique for volume measurement, it is assumed that the isotopic material is homogeneously distributed and that disappearance of the material is negligible or can be measured.

Statistics: A counting rate of a liquid sample is subject to at least 3 sources of error: (1) an error arising from the random nature of the disintegration process; (2) a pipetting error; and (3) a geometrical error resulting from variations in sample containers, the position of samples, etc. The above errors may be expressed as fractional errors, and are employed throughout this study. A fractional error is defined as the standard error divided by the mean, expressed in percentage form. The individual variables of an observed counting rate of a liquid sample may be expressed as a product;

$$R = C \cdot V \cdot G \tag{5}$$

where C is the counting rate per unit volume, V is the sample volume, and G is a geometrical factor. It can be shown that the fractional error of the counting rate (F) will be equal to the square root of the sum of the squares of the fractional errors of the individual parameters.[1] This may be expressed as follows:

$$F = (F_c^2 + F_v^2 + F_g^2)^{1/2} \tag{6}$$

where F_c, F_v, and F_g are the fractional errors associated with C, V, and G, respectively.

Procedure.—Human serum albumin-I^{131} (RISA),* less than 2 weeks old, was employed in all studies. The stock material was diluted to a concentration of 25 μc per milliliter in 30 ml. of normal saline under sterile conditions. To this solution was added 250 mg. of unlabeled human serum albumin. When not in use, both the stock and the diluted solution were kept at 5° C.

Blood and plasma volumes were determined in subjects at bed rest. An intravenous infusion of normal saline or 5 per cent glucose was started into the antecubital vein through a Venopak* venoclysis set to assure the proper entry of the RISA into the vascular system. A B-D Cornwall† constant delivery syringe with a 22 gauge needle was used to deliver 1 to 5 ml. (25 μc) of the diluted RISA into a 2 liter volumetric flask approximately half-filled with tap water. Without changing needles, the syringe was refilled and an identical amount of the original diluted RISA was injected into the soft rubber portion of the Venopak tubing. During delivery, the tubing was clamped immediately above the delivery site. Following delivery, the clamp was removed and the infusion fluid was allowed to flow freely into the patient for at least 5 minutes. Either 2 or 5 ml. B-D syringes were usually employed. The pertinent characteristics of these syringes are given in the results section. Fifteen minutes after the injection of the isotope into one arm, approximately 10 ml. of blood was drawn without stasis from the antecubital vein of the

*Abbott Laboratories, North Chicago, Ill.
†Becton, Dickinson & Company, Rutherford, N. J.

other arm. The sample was delivered into a heparinized tube and 1 or 2 ml. of whole blood was immediately pipetted into a Kimble patent lip glass vial (No. 60900)* for radioactive assay. The remaining portion of the blood sample was centrifuged immediately, and 1 or 2 ml. of plasma was pipetted into a similar vial. A corresponding sample for assay was obtained from the 2 liter standard solution after the dilution had been completed with tap water. Radioactive assay was accomplished in one of the two R-C scientific well-type scintillation counters and all samples were counted sufficiently to assure a 95 per cent error of less than 1 per cent. All pipettes employed in this study were the transfer type either of Exax* or Normax* grade. Blood volume and plasma volume were calculated according to equation 4 of the previous section.

TABLE I. PIPETTING ERROR IN 1 AND 2 ML. PIPETTES

SAMPLE NO.	1 ML. PIPETTE WEIGHT OF CONTENTS (GM.)	2 ML. PIPETTE WEIGHT OF CONTENTS (GM.)
1	0.9994	1.9865
2	0.9977	1.9934
3	0.9990	1.9927
4	0.9982	1.9883
5	0.9954	1.9934
6	0.9953	1.9944
7	0.9985	1.9885
8	0.9963	1.9939
9	0.9988	1.9890
10	0.9993	1.9910
11	0.9982	1.9903
12	1.0007	1.9897
13	0.9977	1.9889
14	0.9983	1.9927
15	0.9960	1.9882
16	1.0008	1.9981
17	0.9999	1.9932
18	0.9964	1.9929
19	0.9977	1.9918
20	0.9969	
Mean	0.9980	1.9914
Standard error	0.0016	0.0027
Pipetting error (F_v)	0.16 per cent	0.14 per cent

RESULTS

Error Evaluation.—

Pipetting error: In order to evaluate subsequent errors, it was first necessary to establish the standard error associated with the pipettes used for the transfer of samples. One and two milliliter volumetric pipettes were tested. Dilute aqueous solutions of RISA were pipetted with the same pipette and with different pipettes into weighed containers. The containers were sealed and reweighed to determine the weight of the contents. The results of two such experiments using different pipettes are given in Table I. In both experiments, the fractional errors were comparable and much less than 1 per cent. In addition, 20 deliveries from a single 1 ml. pipette resulted in a fractional error of 0.11 per cent, while a similar experiment with a 2 ml. pipette gave an error of 0.06 per cent.

*Kimble Glass Company, Toledo, Ohio.

Geometrical error: The fractional error resulting from small variations in sample containers was established by a statistical analysis of counting rate and volume (pipetting error) data. For simplicity, the errors which arise from variation in sample containers were combined with those resulting from variation in placement of the containers in the well counter. A summary of data for this analysis is given in Table II. Independent radioactive assays were obtained on each group of samples and the fractional error due to geometrical variation was calculated for each assay. For 1 ml. volumes, the average fractional error was 0.99 per cent and for 2 ml. it was 0.73 per cent. It should be noted that this error is much greater than the pipetting error. A larger geometrical error was observed with the Kimble screw-cap vial (No. 60910),* which is smaller in size to the vial employed here. In 114 samples of 2 ml. volume, the average geometrical error was 3.44 per cent. This error was considered too large for accurate radioassay procedures.

TABLE II. GEOMETRICAL ERROR ASSOCIATED WITH KIMBLE SAMPLE VIALS CONTAINING 1 AND 2 ML.

EXPERIMENT	NO. OF SAMPLES	VOLUME (ML.)	MEAN TRUE COUNTING RATE (C.P.S.)	EXPERIMENT FRACTIONAL ERROR (F) (PER CENT)	BACKGROUND COUNTING RATE (C.P.S.)	COUNTING RATE ERROR (F_c) (PER CENT)	GEOMETRICAL ERROR (F_g) (PER CENT)
1	20	1	140.6	0.70	20.5	0.18	0.66
2	20	1	140.9	0.77	20.7	0.18	0.73
3	20	1	140.1	1.06	20.5	0.18	1.03
4	20	1	91.6	1.07	12.1	0.18	1.05
5	20	1	91.7	1.47	12.1	0.18	1.46
6	20	2	190.5	0.72	12.3	0.17	0.70
7	20	2	189.8	0.78	12.3	0.17	0.76

TABLE III. THE VOLUMETRIC ERROR ASSOCIATED WITH CONSTANT DELIVERY SYRINGES

EXPERIMENT NO.	SYRINGE SIZE (ML.)	NO. OF SAMPLES	VOLUME (ML.)	MEAN TRUE COUNTING RATE (C.P.S.)	EXPERIMENT FRACTIONAL ERROR (F) (PER CENT)	BACKGROUND COUNTING RATE (C.P.S.)	COUNTING RATE ERROR (F_c) (PER CENT)	SYRINGE ERROR (F_s) (PER CENT)
1	2	10	0.2	51.5	2.62	27.3	0.21	2.42
2	2	10	0.4	103.3	1.80	27.3	0.17	1.49
3	2	10	0.5	55.9	1.47	18.7	0.31	1.04
4	2	10	1.0	111.1	1.71	18.7	0.26	1.37
5	2	10	1.0	210.7	0.49	14.6	0.24	0.00
6	2	4	1.0	55.6	2.37	21.8	0.51	2.09
7	2	10	2.0	207.0	0.92	16.1	0.24	0.51
8	2	4	2.0	100.2	1.49	21.8	0.44	1.22
9	5	10	2.5	508.1	1.17	14.6	0.23	0.88
10	5	4	2.5	104.6	1.50	21.8	0.43	1.24
11	5	4	2.5	125.7	0.40	21.8	0.42	0.00
12	5	10	3.0	315.8	1.32	18.7	0.24	1.07
13	5	10	5.0	900.4	0.94	14.6	0.23	0.55
14	5	10	5.0	452.7	1.29	16.1	0.23	1.04
15	5	4	5.0	222.8	1.66	21.8	0.39	1.44
16	5	4	5.0	187.2	1.63	21.8	0.40	1.40
17	10	10	5.0	239.5	0.65	15.2	0.25	0.00

*Kimble Glass Company, Toledo, Ohio.

Constant delivery syringe error: From the preceding data and appropriate statistical analysis, it is possible to evaluate the error associated with the constant delivery syringe over a wide range of volumes. Direct deliveries of dilute RISA solution were made into the sample containers from syringes. In those experiments where the delivered volume was less than 1 ml. water was added to attain this volume. In general, the volumes of most consequence to this study fall between 1 and 2 ml. However, additional data on other volumes were obtained to determine the usable range of the syringes. The results are summarized in Table III. The calculated fractional errors are highly variable but the maximum error (2.42 per cent) is associated with the minimum volume (0.2 ml.) delivered. The average error from 0.2 ml. to 5 ml. using 3 different sized syringes was 1.04 per cent. In 3 experiments (Nos. 5, 11, and 17) the syringe error is reported as zero since the sum of the squares of the counting rate and geometrical errors exceeded the observed experiment error. The errors associated with volumes greater than 2 ml. were calculated using the geometrical error decreases with larger samples, the estimated errors given here are probably high.

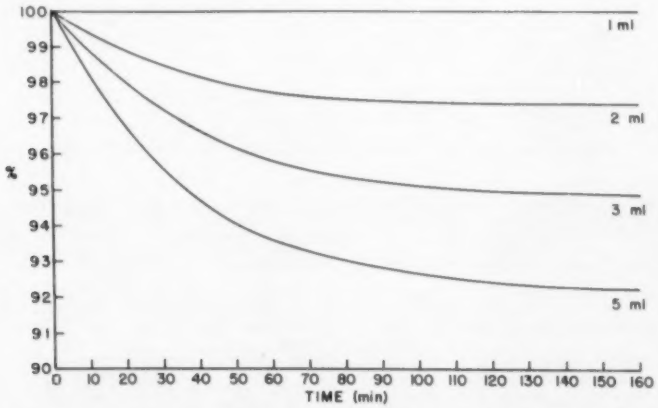

Fig. 1.—The effect of red cell sedimentation upon counting rate. The ordinate represents the percentage of original activity (zero time, no sedimentation). Values plotted here are for normal blood and are the mean results obtained from a recording counting rate meter. Curves for 4 different sample volumes are shown.

Settling error: When whole blood containing RISA separates upon standing, the majority of the radioactivity will be found in the plasma layer. The geometry of counting may be altered if the settling process occurs during the radioassay procedure. The influence of this process upon the counting rate in normal blood is shown in Fig. 1. Although the effect is considerable in larger samples, it is negligible in volumes below 2 ml., especially if the time of assay is less than 5 minutes. There is an additional error due to red cell sedimentation. If red cells are allowed to settle and then mixed for redistribution, the original distribution is difficult to reproduce. In practice, this is avoided by the immediate pipetting of blood into the sample vial.

Absorption and scatter error: Blood or plasma may be expected to exhibit different scattering and absorption properties than water. These properties would also be expected to vary as a function of the volume of the sample. Accordingly, samples of whole blood and water (1 to 5 ml.) containing identical amounts of RISA were assayed. The results are shown in Fig. 2. Although the decrease in the efficiency of counting with respect to volume is appreciable, the difference in the counting rates of blood and water is negligible below 2 ml.

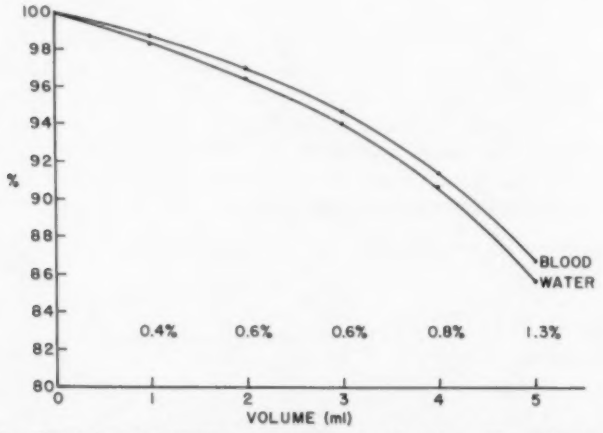

Fig. 2.—Comparison of counting rates in blood and water. The ordinate represents the percentage of activity measured in a negligible volume (0.01 ml.) and the abscissa represents the sample volume. Curves are shown for activity in both blood and water and each point is the mean of 8 measurements. The difference in observed counting rate between blood and water is given by the percentage volumes along the abscissa.

TABLE IV. THE LOSS OF RISA ON GLASS UNDER VARIOUS CONDITIONS. PERCENTAGE VALUES REPRESENT THE FRACTION OF ACTIVITY AT ZERO TIME WHICH HAS NOT DISAPPEARED FROM SOLUTION

EXPERIMENTAL CONDITIONS (2-LITER VOLUMETRIC FLASKS)	TIME (MIN.)							
	0	10	20	30	40	50	60	120
RISA + distilled H_2O—continuous mixing	100	96	93	90	88	86	85	82
RISA + distilled H_2O—initial mixing only	100	98	96	94	93	92	92	91
RISA + distilled H_2O + albumin (0.1 mg./ml.)—continuous mixing	100	100	100	100	100	100	100	100
RISA + tap water—initial mixing only	100	99	98	97	97	97	97	97
RISA + tap water + albumin (0.01 mg./ml.)—initial mixing only	100	100	100	100	100	100	100	100

Loss of radioalbumin on glassware: Radioalbumin in dilute solution accumulates on glass surfaces.[2] This may be demonstrated by the continuous flow of dilute RISA through a glass container within a well counter. The results of such an experiment are shown in Fig. 3. In brief, these losses may be minimized or eliminated by: (1) using RISA with a low specific activity; (2) reducing the amount of mixing; (3) reducing of time of exposure of RISA to glass; (4) adding ions; (5) using solutions with pH values other than the

isoelectric point of albumin; and (6) working at low temperatures. The effects of the first 4 listed above are shown in Table IV. The effect of ions and pH will be the subject of another report.

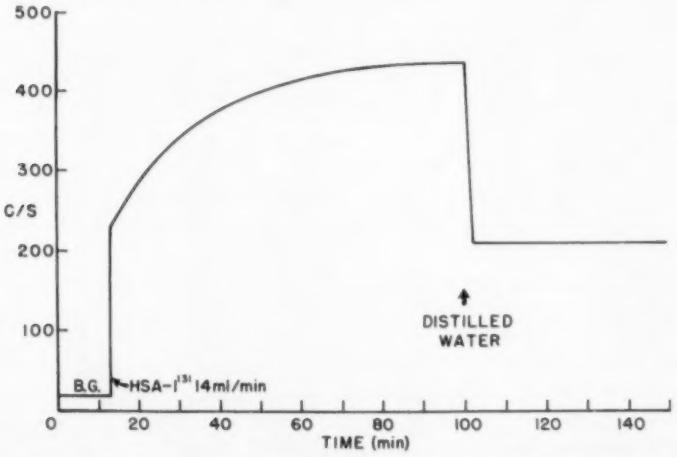

Fig. 3.—The accumulation of HSA-I^{131} on glass (pH 4.8). An aqueous RISA (HSA-I^{131}) solution was allowed to flow through a glass container within a well counter. The counting rate was continuously recorded and continued to rise as the RISA accumulated on the glass. After flushing the system with distilled water, the counting rate did not return to the background (B.G.) level.

Normal Blood Volume Data.—The modified method (including precautions to eliminate the above errors) was employed to measure blood and plasma volumes in 50 hospitalized, ambulatory patients who were convalescing from minor surgical procedures or simple fractures. These patients showed no evidence of cardiac, renal, or hepatic disease. Table V contains the results observed in this group. The mean blood volume for the group was 77.3 ± 9.2 ml. per kilogram of body weight. The data were recalculated on a basis of ideal body weight (Metropolitan Life Insurance Co. Tables) and this resulted in a mean value of 76.4 ± 9.0 ml. per kilogram which was not significantly ($p = 0.60$) different from the value calculated with actual weight as a reference. The mean plasma volume was 41.8 ± 6.5 ml. per kilogram, and the mean hematocrit (calculated from blood and plasma volumes) was 46.0 ± 4.0 per cent.

DISCUSSION

A method may yield measurements which are reproducible or which are comparable to those obtained by other methods, yet the actual error may be considerable. There are numerous reports of methods for measuring blood volume with radioalbumin showing the reproducibility of the method and agreement with measurements resulting from other techniques,[3-6] but there is a paucity of information concerning the technical accuracy of the methods.

The present study is a systematic attempt to evaluate the technical errors of a modified method for blood volume determination using RISA, although these errors are common to most methods.

TABLE V. BLOOD AND PLASMA VOLUME DATA IN NORMAL MEN LIVING AT AN ALTITUDE OF 1 MILE ABOVE SEA LEVEL

SUBJECT	AGE (YR.)	WEIGHT (KG.)	BLOOD VOLUME (LITERS)	PLASMA VOLUME (LITERS)	HEMATOCRIT (PER CENT)	BLOOD VOLUME (ML./KG.)	PLASMA VOLUME (ML./KG.)
H. B.	20	80.0	6.16	3.25	47.2	77.0	40.6
W. J.	21	71.8	4.83	2.58	46.6	67.3	35.9
E. B.	21	76.7	5.87	3.11	47.1	76.5	40.6
B. M.	23	61.4	5.61	3.16	43.6	91.4	51.5
E. W.	23	50.0	4.32	2.26	47.7	86.4	45.2
D. J.	23	59.0	5.12	2.68	47.6	86.8	45.4
L. M.	24	68.6	4.70	2.41	48.7	68.5	35.1
M. U.	24	68.4	5.77	3.13	45.7	84.4	45.8
K. A.	24	70.0	5.47	3.11	43.1	78.1	44.4
I. B.	24	84.1	5.93	3.15	46.8	70.5	37.5
S. P.	25	57.7	3.35	1.71	49.1	58.1	29.6
J. S.	25	54.7	5.06	2.97	41.3	92.5	54.3
H. H.	25	78.2	5.13	2.88	44.0	65.6	36.8
L. P.	26	72.4	5.48	2.62	52.2	75.7	36.2
G. C.	27	69.7	5.15	3.11	39.7	73.9	44.6
P. D.	27	73.0	5.65	3.10	45.1	77.4	42.5
J. F.	27	69.1	4.73	2.79	41.0	68.5	40.4
B. C.	28	73.6	4.66	2.48	46.8	63.3	33.7
J. G.	28	74.8	5.42	2.94	45.6	72.5	39.3
R. C.	28	72.3	5.46	3.35	38.7	75.5	46.3
D. V.	29	81.8	5.29	2.90	45.2	64.7	35.5
L. M.	29	55.5	4.67	2.41	48.5	84.1	43.4
R. B.	30	89.6	6.20	3.20	48.4	69.2	35.7
J. M.	30	79.4	6.84	3.29	51.9	86.2	41.4
G. U.	32	63.2	4.34	2.50	42.5	68.7	39.6
J. P.	33	80.0	6.14	2.93	52.2	76.8	36.6
G. I.	33	80.2	5.76	3.11	45.9	71.8	38.8
W. P.	34	57.1	4.55	2.26	50.4	79.7	39.6
D. C.	34	65.5	5.29	3.11	41.2	80.8	47.5
C. M.	34	65.9	6.02	3.12	48.2	91.4	47.3
T. M.	35	55.9	4.41	2.20	50.1	78.9	39.4
B. L.	35	63.2	4.97	2.54	48.9	78.6	40.2
P. F.	36	51.8	4.28	2.62	38.8	82.6	50.6
R. R.	37	59.6	3.72	1.94	48.0	62.4	32.6
B. McK.	37	78.9	4.96	2.58	48.0	62.9	32.7
L. I.	37	68.5	4.67	2.13	54.4	68.2	31.1
E. W.	37	95.5	6.76	3.27	51.6	70.8	34.2
J. E.	38	72.1	6.50	3.31	49.1	90.2	45.9
H. F.	38	63.2	5.43	3.03	44.2	85.9	47.9
C. A.	39	51.8	4.22	2.71	35.7	81.5	52.3
J. C.	42	69.6	4.48	2.27	49.3	64.4	32.6
D. C.	42	63.4	5.40	3.29	39.1	85.2	51.9
A. T.	42	58.2	5.11	2.73	46.6	87.8	46.9
C. R.	43	75.0	6.98	4.01	42.6	93.1	53.5
O. R.	44	59.3	4.60	2.41	47.5	77.6	40.6
T. A.	44	62.7	5.86	3.51	40.1	93.5	56.0
J. M.	47	60.5	5.41	2.92	46.0	89.4	48.3
J. L.	61	61.3	4.91	2.68	45.4	80.1	43.7
R. C.	61	83.2	5.86	3.00	48.8	70.4	36.1
J. McC.	61	78.2	5.89	3.23	45.3	75.3	41.3
Mean					46.0	77.3	41.8
Standard error					4.0	9.2	6.5

The findings indicate that the technical errors of this method are small. The largest error is that associated with the constant delivery syringe. Although the results for different volumes were variable, the maximum error

was observed with minimum volumes. In order to minimize the syringe error, a volume of 1 ml. in a 2 ml. syringe or 2 to 3 ml. in a 5 ml. syringe should be used.

Without additional expense for special containers, very little can be done to reduce the error due to geometry. Larger sample volumes for radioassay would be expected to result in less error; however, 2 ml. samples manifest a small error and are easily obtained.

Pipetting error is insignificant if high quality pipettes are employed. It is surprising that this error is so small even in the hands of different technicians working under routine conditions. The average absolute error (i.e., deviation from a given pipette volume) is less than 0.25 per cent and this is less than the pipette tolerance recommended by the National Bureau of Standards.[7]

It has been shown here that although errors due to red cell sedimentation, absorption, scatter, and loss of radioalbumin on glassware can be appreciable, they can be eliminated by simple precautions. The settling of red cells in the sample vial is obviously a function of hematocrit and sedimentation rate; thus greater errors would probably occur in disease states where these variables were altered. In such cases the error can be kept at a minimum by using higher doses of radioactivity so that smaller sample volumes and shorter time intervals for radiocounting can be employed. The use of low specific activity RISA eliminates the error due to the loss of radioalbumin on glass. In addition, it minimizes the probability of protein alteration due to radiation.[8]

With appropriate statistical analysis[1] using the individual fractional errors, it is possible to calculate a fractional error for the entire technique. This was found to be 2.0 per cent or approximately 1.5 ml. per kilogram in the average blood volume of the male. This error is much less than the observed error in blood volume for the group of normal men (Table V). Thus, with the method described here, the variability in the measured blood volume cannot be attributed to technical errors alone. Indeed, it is apparently accurate enough to measure physiologic variation.

The normal blood and plasma volumes recorded here at one mile above sea level are elevated, but not significantly ($p > 0.15$) different from those reported near sea level.[3] In addition, they are not significantly ($p = 0.50$) different from values obtained with T-1824 dye at an altitude of one mile.[9] Since blood and plasma volumes are measured, it is possible to compute a hematocrit which should approximate the venous hematocrit. The mean hematocrit was significantly ($p = 0.04$) higher than a corresponding mean value obtained near sea level.[3] This increased hematocrit is similar to that reported by others at this altitude.[9]

SUMMARY

A modified technique for measuring blood and plasma volume with RISA has been presented. The technical errors of this technique have been evaluated and minimized. Data on 50 normal men living at an altitude of 1 mile

are given. It is concluded that the modified method has a low degree of technical error (± 2 per cent standard error) and it is suitable for routine clinical use.

We are deeply indebted to Drs. Harold Elrick and Joseph H. Holmes for their helpful comments and criticisms.

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A MICROMETHOD FOR DETERMINING CLOTTING TIME, USING CAPILLARY BLOOD AND SILICONIZED TUBES

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MANY methods have been devised to determine the clotting time of whole blood, the most widely used being variations of the Lee-White procedure which necessitate a venipuncture. The Lee-White[1] method and its modifications have the advantage of accuracy, reproducibility, and a backlog of years of clinical experience.

While studying the response of patients to concentrated aqueous heparin preparations, it became necessary to perform as many as 6 venipunctures on individual patients in a space of several hours. To eliminate these repeated venipunctures, a new method was devised which was better tolerated by the patients and yet of sufficient accuracy to permit its use for careful clinical studies. It was found that by siliconizing capillary tubes, capillary blood could be used in a simple, reproducible manner to measure clotting time and heparin response. The use of capillary tubes to determine cloting times of blood was first described by Sabrazes in 1899.[2] However, although the method is convenient, its lack of sensitivity heretofore rendered it unsatisfactory for exact clinical studies.

MATERIALS AND METHODS

The capillary tubes used were 150 mm. long, thin-walled coagulation tubes, outside diameter, approximately 0.5 mm.† So-called melting point tubes varied considerably in diameter, gave erratic results, and could not be adapted to the method.

All procedures for preparing the tubes were carried out in the original glass container provided by the manufacturer. The tubes were first rinsed with 0.1 N sodium hydroxide for a 10- to 15-minute interval, followed by 3 rinsings in distilled water. After draining away the water, they were immersed for a few minutes, with frequent tilting in a 1:100 solution of aqueous silicone.‡ They were again rinsed 3 times in distilled water, and dried in the oven at 80° C. for 3 to 4 hours. After the application of the silicone, the tubes could be used at any desired interval.

For drawing the blood, a plastic infant feeding tube§ was placed over one end of the capillary tube and blood was aspirated by mouth suction to minimize contact of the blood with air and to facilitate filling.

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*Josiah Macy Student Fellow.

†The Harshaw Chemical Company, Cleveland, Ohio.

‡Siliciad, Clay-Adams, Inc., New York, N. Y. For a more exact end point, the pH of the solution should not be adjusted with ammonium hydroxide.

§Size 8 French, the Pharmaseal Laboratories, Glendale, Calif.

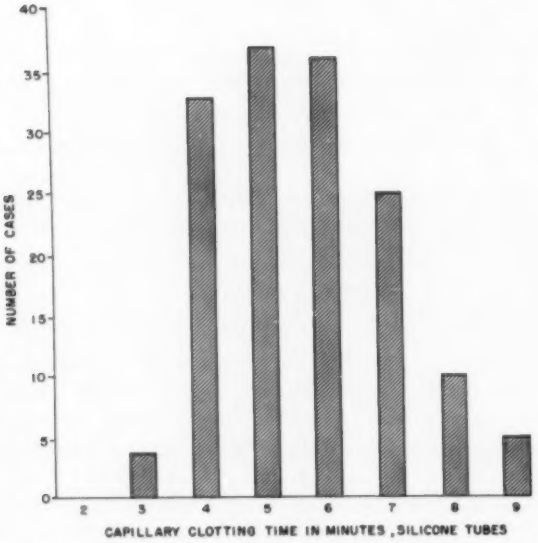

Fig. 1.—Clotting times in minutes as determined by the silicone capillary tube method in 150 normal adult subjects.

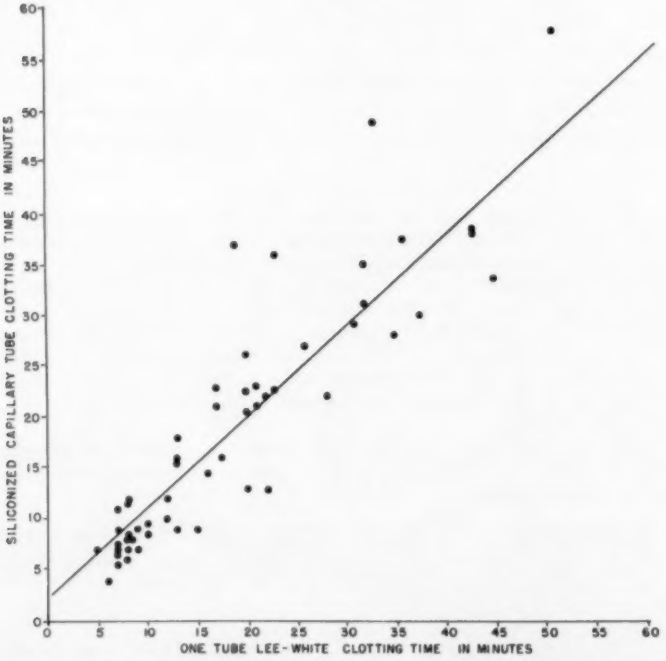

Fig. 2.—Clotting times simultaneously determined by silicone capillary tube and one tube Lee-White methods in 55 subjects receiving heparin. The values plotted were obtained at intervals varying from 10 minutes to 4 hours after intravenous administration of the drug.

The finger tip was first wiped thoroughly with an alcohol swab and allowed to dry. The puncture* was made while compressing the donor's finger firmly between the technician's thumb and index finger. Pressure was applied across the distal phalanx until the finger became plethoric. The puncture was performed with continuing pressure. The first drop of blood was utilized for the test, since it was found that subsequent drops gave shorter clotting times. Christie[3] attributed this shortening to the higher concentration of tissue thromboplastin and platelets in subsequent drops. Although firm digital pressure was continued while blood was being drawn, "milking" of the finger to obtain blood was avoided, since this caused a marked shortening in the clotting time. The stop watch utilized for timing was started as soon as the puncture was effected. If blood did not flow freely, a second adjacent puncture was made immediately; this procedure was found to give a more consistent clotting time than "milking" the finger tip. An inadequate puncture, without free flow of blood, proved to be the largest single source of error in the method. The tip of the capillary tube was placed at the puncture site, and blood was drawn to an approximate height of 75 mm. in the tube.

The capillary tube was then held in the closed fist to maintain a constant temperature of approximately 34° C. At 1-minute intervals, the tube was tilted vertically until blood no longer flowed. Subsequently, the tube was broken every 30 seconds between thumb and forefinger until a fibrin thread was seen between the broken ends. The tube was always broken at the end opposite to that at which the blood entered. Appearance of the fibrin thread determined the end point. If the clotting time was prolonged, as in patients receiving heparin, the tube was broken less often, usually every 1 to 2 minutes, in order to conserve the length of glass. With greatly prolonged clotting times, it was sometimes found that the blood did not clot uniformly throughout the tube, initially clotting at the air-blood interfaces and later toward the center of the column. In such instances, the end point was then arbitrarily taken as the formation of two successive fibrin threads.

Although the original Lee-White method[1] has been modified to measure coagulation in 2 or more tubes, previous studies from this laboratory have shown that the one tube method was as accurate as the more time-consuming multitube procedure, provided that certain precautions were scrupulously observed.†[4] It was shown that in the case of a 3 tube method there was a constant relationship between the clotting time in tubes 1, 2, and 3, and that after administration of heparin the relative increase in coagulation time in any 1 of the 3 tubes was constant. The one tube Lee-White procedure, therefore, was used as a basis of comparison with the capillary method. In performing the one tube procedure, 4 ml. of blood was obtained by venipuncture in a clean dry syringe with a minimum of trauma to the vein, the needle was removed, and 1 ml. of the blood placed in a scored 13 by 100 mm. test tube. A stop watch was then started. The tube was gently tilted at 1-minute intervals. Coagulation was assumed to be complete when the tube could be inverted without displacing the clot. The stop watch reading at this point determined the clotting time.

RESULTS

Clotting times of 150 normal adult subjects (75 men and 75 women), grouped at 1-minute intervals, are shown in Fig. 1. The majority of the values fall in the range of 4 to 8 minutes, with a mean of 6.0 minutes and a standard deviation of 1.45 minutes. No significant differences were observed between men and women.

In order to compare the present method with the one tube Lee-White method, blood samples were taken simultaneously from venipuncture and from finger puncture by two technicians. Each determined the clotting time independently by the selected method. The values, determined separately, were

*The most satisfactory lancets for this purpose were obtained from Becton, Dickinson and Company, Rutherford, N. J.

†Normal values for one tube method: 8.9 minutes ± 2.2.

compared after completion of the test. Fig. 2 shows 55 pairs of these simultaneously determined clotting times. The values plotted were obtained at intervals varying from 10 minutes to 4 hours after administration of intravenous heparin.* The similarity between the methods is apparent and is highly significant statistically.†[5]

Fig. 3 shows the response to the intravenous administration of 50 mg. of heparin to 4 normal subjects. The clotting times, determined simultaneously by the two methods, were determined before, 45, 90, and 150 minutes after the injection of the heparin. The similarity of the two methods is again apparent.

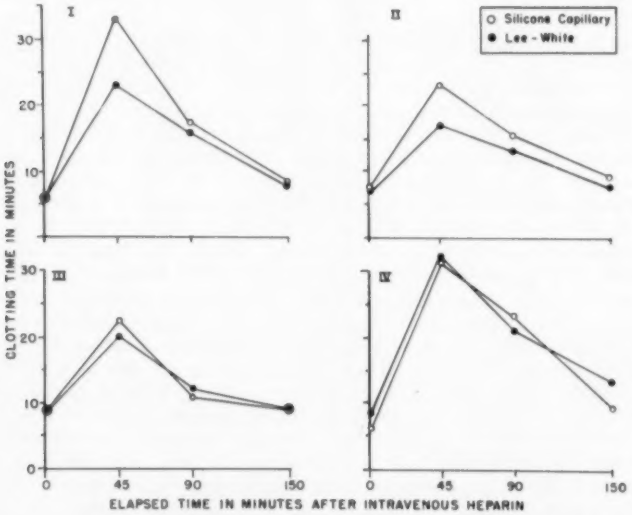

Fig. 3.—Response to intravenous heparin determined simultaneously by the one tube Lee-White and silicone capillary tube methods in 4 normal subjects. The clotting times were determined before, 45, 90, and 150 minutes after injection of the drug.

DISCUSSION

Our data have indicated that there is a good correlation between values obtained by the capillary and the Lee-White methods throughout the usual range of clotting times encountered in heparin therapy. As ordinarily performed, clotting times on capillary blood are quite short, inaccurate, and relatively insensitive in measuring heparin response. For this reason little reliance has been placed on their use, and even recently developed micromethods have entailed the use of venous blood.[6-8] This has put a deterrent on the frequent determination of heparin response.

*Aqueous heparin sodium. 5,000 U.S.P. units (50 mg.).

†Equation of regression line: $y = 0.925x + 1.88$; $b_{yx} = 0.925$; $b_{xy} = 0.851$; $r = 0.887$; t for regression $yx = 14.002$, $p < 0.001$.

With the development of slowly absorbed, concentrated heparin, an accurate micromethod for frequent determinations of blood coagulation has obvious advantages. We believe that the present method is sufficiently sensitive and accurate for such purposes. Until further clinical evidence is available, the method should not be substituted for the Lee-White clotting time in patients with coagulation disorders.

SUMMARY

A new method of performing whole blood clotting times has been presented. This method utilizing capillary blood and siliconized capillary tubes obviates the necessity of obtaining venous blood. The mean value in 150 normal subjects was found to be 6.0 minutes with a standard deviation of 1.45 minutes. The method, which shows a close correlation with the one tube Lee-White method, has been found to be of value when repeated determinations of clotting time are required, particularly with the prolonged use of heparin.

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NEUTRON ACTIVATION ANALYSIS IN THE DETERMINATION OF BONE SODIUM

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SODIUM has been a known constituent of bone since at least 1894 when Gabriel discussed its occurrence there.[1] Following the work of Harrison and associates[2] on tissue electrolyte analysis, the significance of "extra" bone sodium began to gain appreciation. Estimation that up to 40 per cent of the total body sodium stores may be in cortical bone[3, 4] lent further import to this reservoir. The availability of bone sodium for exchange has been demonstrated isotopically by several groups[3, 4] and inferentially by Bergstrom.[5] Interesting conjectures regarding the role of bone sodium during adrenal insufficiency have been postulated.[6]

Analysis for bone sodium has proved somewhat difficult by previous techniques, with different authors claiming varying accuracy for their procedures. The calcium : sodium ratio of 40 : 1 makes direct flame photometry on bone digests impractical because of the interference of the calcium spectrum, and thus some manner of separation of the two cations has proved necessary prior to analysis. Methods for this separation have been of two types; repeated precipitation of calcium by the addition of oxalate[7] and, more recently, separation of calcium by the use of cation exchange chromatography.[8, 9] The former method is complicated by some inevitable coprecipitation of sodium with calcium oxalate; the latter, although apparently capable of achieving good separation, is time consuming. In both methods, it is necessary to digest the bone specimen prior to analysis.

Neutron activation analysis remains a somewhat unexplored biologic technique. With this method, the sample is placed in a nuclear reactor and is exposed to a flux of thermal neutrons. By neutron capture, artificial radioactivity is produced in the exposed specimen. Differences in emission, half-life, and other physical characteristics make analysis of the radioactive and, hence, ionic components possible. Sodium^{23}, by virtue of its large neutron capture cross section, is readily activated to Na^{24}. Calcium is also activated, but the only isotope produced in appreciable quantities is Ca^{49}, with a short half-life of 8 minutes. Predicted activities for bone tissue, based on the irradiation of one gram of bone at a flux of 10^{12} neutrons per square centimeter per second for 10 minutes

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The opinions herein expressed are those of the authors and do not necessarily represent the opinion of the United States Navy.

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(and assuming a sodium concentration of 0.2 mEq. per gram and a calcium concentration of 8 mEq. per gram) give the activities shown in Table I. Neither potassium[42], chlorine[38], nor phosphorus[32] may be expected to contribute significant activity to these totals. Thus, the determination of bone sodium despite the preponderance of calcium should be entirely feasible by neutron activation analysis, provided an appropriate period is observed for the decay of Ca^{49}.

TABLE I

TIME (AFTER ACTIVATION)	SODIUM[24] (μc)	CALCIUM[49] (μc)
0	12	190
1 hour	11.4	16.1
2 hours	10.8	0.08

METHODS

Cortical human long bone specimens were used throughout; these were obtained from sterile autopsies, and stored after freeze-dry preparation.* The minimal marrow tissue that was visibly adherent was removed insofar as possible, to avoid the high sodium concentration present in the vascular marrow. Samples were analyzed in both the solid and digested state. For the latter, preparation was as follows: 12 hours ashing at 600° F., followed by solution in 6 M HNO_3. An aliquot containing 15 per cent by weight was irradiated. Specimens analyzed following digestion ranged from 2.1 to 2.9 Gm.; specimens analyzed without any preparative measures ranged from 0.3 to 1.8 Gm. The bone tissue was sealed into polyethylene containers after weighing (and dilution in the case of digested samples), and was irradiated for 10 minutes at a flux of approximately 10^{12} neutrons per square centimeter per second. Relative counting was performed, and no attempt was made to quantitate the neutron flux to greater accuracy. A weighed amount of NaCl was activated with each specimen, and was placed adjacent to the specimens in order to receive the same flux within the nuclear reactor. Final quantitation of the sodium content was based on the formula:

$$W_u = \frac{(CPM_u) \times W_k}{(CPM_k)}$$

where W_k is weight of Na in standard; W_u is weight of Na in bone; CPM_k is counts for standard; CPM_u is counts for bone. Calculation by this method obviates any decay correction, if bone and standard are counted at the same time. Counting was performed in a well-type gamma scintillation counter which afforded constant geometry in volumes less than 3 ml. Quantitation was based on the 2.7 Mev. peak of Na^{24}. Gamma ray spectroscopy was performed on each specimen to ensure that the spectrum present was that of Na^{24}, and half-life determinations provided further evidence of this fact. No attempt at beta counting was made.

The only unusual care which must be observed is in regard to preparation of the plastic containers; after sealing they must not be handled prior to activation, for the sodium in perspiration is also activated, and will distort the values obtained. The containers are acid washed before and after activation, and are irradiated while encased in a separate plastic bag.

A minimal interval of 3 hours elapsed between activation and counting, and, during this time, the short half-lived isotopes of calcium have decayed to uncountable values.

RESULTS

The sensitivity of this method was illustrated by obtaining a pure Na^{24} spectrum after activating a $CaCO_3$ specimen known to contain less than 0.5

*Courtesy of the Tissue Bank, U. S. Naval Medical School, Bethesda, Md.

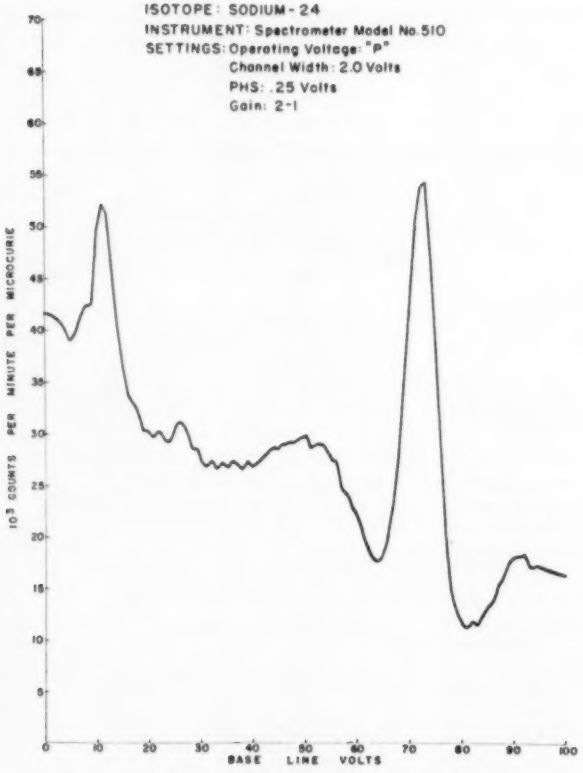

Fig. 1.—Illustration of the gamma spectrum of sodium[24].

TABLE II

SAMPLE	WEIGHT	mEq. Na/GM. BONE	DIGEST OR SOLID	
1	1.794	0.236	Solid	$\bar{x} = 0.294$
	0.567	0.325		$\sigma = 0.050$
	0.586	0.322		R.E. = 17%
2	2.169	0.280	Digest	
3	0.389	0.256	Solid	$\bar{x} = 0.265$
	0.537	0.281		$\sigma = 0.013$
	0.358	0.260		R.E. = 5%
4	2.879	0.154	Digest	$\bar{x} = 0.164$
	2.175	0.174		$\sigma = 0.010$
	2.354	0.165		R.E. = 6%
5	0.698	0.214	Solid	$\bar{x} = 0.236$
	0.314	0.256		$\sigma = 0.021$
	0.322	0.238		R.E. = 9%
Mean	1.164	0.243		
$\sigma = 0.054$				
R.E. = 22%				

per cent sodium by weight. Fig. 1 illustrates the characteristic gamma spectrum obtained from Na^{24}.

Table II gives the data obtained from neutron activation analysis, as performed on 5 different bones.

Flame photometry was performed on random samples of bone, utilizing a digest technique as outlined above. Values so obtained ranged from 0.361-0.399 mEq. per gram of bone; there was no correlation with data as obtained from neutron activation analysis. No attempt was made at oxalate precipitation or at ion-exchange column separation.

DISCUSSION

Prior experience in this laboratory using neutron activation analysis of biologic material has shown that sodium, in most situations, is so readily activated that its separation, if other ions are to be analyzed, becomes mandatory. Analysis for trace materials is rendered impossible by the dominance of the sodium spectrum, and, if gamma counting is to be done, ion exchange methods for the separation of sodium must be performed. In the present instance, the problem is just the reverse, and activation analysis can be easily performed for sodium in the face of a 40:1 gradient for calcium over sodium.

The major advantage inherent in this technique is that no preliminary separation or preparation need be performed on the bone specimen. Using the described technique the bone may be used for other determinations about 60 hours following activation, and it is available in the intact form.

Objection might be raised that the activation of standard (Na) and of bone (Na, Ca, and P) are not equivalent, and that the presence of calcium and phosphorus in bone lowers the effective flux making standard and sample not subject to equivalent neutron flux. Fortunately, the neutron cross section for both calcium and phosphorus is low, and on theoretical grounds one would not expect marked deviation. Adding calcium to a constant amount of sodium, in gradients up to 40:1 (Ca:Na) did not result in any observable change in the activation of sodium, and thus the objection seems not applicable to this situation.

TABLE III

PRECURSOR ISOTOPE	n, p	n, 2n	n, ALPHA
Na^{23} (100%)	Ne^{23}*	Na^{22}†	F^{20}*
P^{31} (100%)	Si^{31}‡	P^{30}*	Al^{28}*
Ca^{40} (96.9%)	K^{40}†	Ca^{39}*	A^{37}†
Ca^{42} (0.64%)	K^{42}‡§	Ca^{41}†§	A^{39}†§
Ca^{43} (0.14%)	K^{43}‡§	Ca^{42}‖	A^{40}‖
Ca^{44} (2.1%)	K^{44}*	Ca^{43}‖	A^{41}‡
Ca^{46} (0.0032%)	--	Ca^{45}†§	--
Ca^{48} (0.18%)	--	Ca^{47}†§	--

*Half-life too short to be present at 3 hours.
†Half-life too long to undergo significant build-up during 10-minute activation.
‡Gamma energy would not be present at 2.7 mEv. counting peak.
§Precursor (naturally occurring) isotope abundance is insignificant.
‖Isotope is stable.

Considerations of fast neutron reactions have been avoided on several grounds. Gamma ray spectroscopy and half-life determinations gave no

evidence of significant amounts of contaminating radioisotopes. Table III illustrates the possible isotopes formed by interaction with fast neutrons. Thus, on the basis of no observed contaminating radioisotopes, plus the predicted production in Table III, fast neutron reactions will probably not interfere with thermal neutron activation.

Five different samples of human cortical bone were analyzed; in all but one, multiple specimens from the same bone were used. Mean value for the entire group was 0.243 mEq. of sodium per gram of dry bone, with a range of 0.154 to 0.324 mEq. Na per gram bone. Standard deviation for the 5 specimens was 0.054, giving a relative error (R.E.) of 22 per cent. Analyzing the data for the separate bone specimens shows that the relative error for the 4 groups of multiple samples ranged from 5 to 17 per cent, indicating a reasonable reproducibility for the data.

Published data on the sodium content of cortical bone are not dissimilar to this value.[10, 11]

Neutron activation analysis may be similarly applied to other materials for the analysis of sodium; this method is not likely to prove popular unless circumstances prevail which make flame photometry or destruction of the sample impossible. Analysis for ions other than sodium proves far more difficult than the above-described method.

SUMMARY

Neutron activation analysis techniques have been applied to the determination of bone sodium. Because of the physical characteristics of sodium, it may be easily determined, even in the presence of an abundance of calcium. The technique and results are described, as performed on freeze-dry human cortical bone specimens.

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STABLE TISSUE CULTURE MEDIA PREPARED IN DRY FORM

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CHEMICALLY defined media used in tissue culture are not only time-consuming to prepare, but frequently contain ingredients which deteriorate when in solution even at a temperature of 4° C. The preparation of media thus becomes a problem of considerable practical importance, particularly when many are required for strains of cells with diverse nutritional requirements or for experimental purposes. In certain long-term experiments it is desirable to employ solutions of constant composition throughout, but in practice this is not readily achieved even when special precautions are taken to purchase chemicals in large quantities. Furthermore, these difficulties are not adequately circumvented in the conventional research laboratory by the preparation of large lots of even the more stable solutions because of limitations of refrigeration space, problems of microbial contamination, or because the technical difficulties encountered in filtration-sterilization are usually proportional to the quantity of fluid involved. Similarly, the use of concentrated solutions is frequently unsatisfactory because of the limited solubility of many of the ingredients. As a consequence of these considerations, studies were initiated to examine the feasibility of mixing ingredients in the solid form and storing these until required for use. This report describes the preparation of uniform mixtures which can be stored for indefinite periods and their use in tissue culture media.

Medium S103 (see Table I for composition) is discussed as a type example. S103 is prepared in the powdered form as follows. The ingredients for 40 liters with the exception of cystine are placed in a porcelain ball mill jar of 1.25 quart capacity which is filled to about one-third its volume with flint "pebbles" 0.5 to 1 inch in diameter. The jar is placed overnight in a vacuum desiccator evacuated to approximately 0.005 mm. Hg by means of a suitable pump. *As a safety precaution, Pyrex glass desiccators should be covered with a desiccator guard whenever vacuum is applied.* After desiccation, the cover (fitted with rubber gasket) is replaced on the ball mill jar and it is rolled overnight. There are several types of machines available commercially which are suitable for this purpose. The resulting homogeneous powder is separated from the flint "pebbles" by passage through a stainless steel sieve. Appropriate quantities of powder are dispensed *immediately* into a series of screw-cap reagent bottles (wide-mouth, amber color) which can be stored in a desiccator

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at 4° or −20° C. Alternately, the desiccator can be eliminated by sealing the bottles with plastic tape* or by the use of the recently developed caps fitted with a polyethylene inner-seal.† It should be emphasized that if traces of residual water are not removed from the chemicals prior to grinding, the mixture frequently becomes caked on the sides of the ball mill jar resulting in an unsatisfactory preparation. Similarly, the finished powder is hygroscopic and if not protected from moisture, it will invariably become caked (consistency of concrete); also, the mixture becomes alkaline when moist as a result of loss of CO_2.

TABLE I. COMPOSITION OF S103

COMPOUND*	CONCENTRATION (mM.)	GRAMS PER 40 LITERS	
Calcium chloride (2 H_2O)	1.8	8.0	(9.3)
Glucose	5.6	40.0	
Magnesium sulfate (7 H_2O)	0.8	4.0	(8.0)
Phenol red (sodium salt)	0.02	0.40	
Potassium chloride	5.5	16.0	
Sodium chloride	116.2	272.0	
Sodium bicarbonate	26.2	88.0	
Sodium monobasic phosphate • H_2O	1.2	5.6	
Alanine†	0.3	1.07	
Aspartic acid	0.15	0.80	
Arginine (HCl)	0.8	5.57	(6.74)
Cystine (HCl)‡	0.05	—	
Histidine (HCl • H_2O)	0.05	0.32	(0.42)
Hydroxyproline	0.15	0.79	
Glycine	0.15	0.45	
Glutamine§	2.0	11.69	
Isoleucine	0.2	1.04	
Leucine	0.2	1.04	
Lysine (HCl)	0.2	1.16	(1.48)
Methionine	0.1	0.60	
Phenylalanine	0.1	0.64	
Proline	0.15	0.69	
Serine	0.2	2.10	
Threonine	0.4	1.92	
Tryptophan	0.05	0.40	
Tyrosine	0.1	0.72	
Valine	0.4	1.87	
Choline chloride	0.01	0.056	
Folic acid	0.005	0.088	
Calcium pantothenate	0.005	0.048	
Mesoinositol	0.01	0.086	
Nicotinamide	0.005	0.024	
Pyridoxal (HCl)	0.005	0.034	(0.040)
Riboflavin	0.001	0.015	
Thiamin (HCl)	0.005	0.060	(0.068)
Streptomycin sulfate	0.008	0.50	

*Medium also contains 100 units of penicillin G per milliliter. This is added to the final solution just prior to use from a 20,000 unit per milliliter stock which is stored at −20° C.

†All amino acids are of L-configuration.

‡Cystine is always omitted from S103 powder and it is added to final solution from a 5 mM. stock in 0.025 N hydrochloric acid which is stored at room temperature.

§When glutamine is omitted from the S103 powder it is added to final solution from a 200 mM. stock which is stored at −20° C.

Solutions are prepared from the powdered-mixture as follows: The powder is added stepwise to the appropriate quantity of water which is stirred by

*Pressure Sensitive Tape No. 471; Minnesota Mining & Manufacturing Co., St. Paul, Minn.

†Poly-Seal caps manufactured by Latchford-Marble Company, Los Angeles, Calif.

means of a magnetic stirrer (the metal stirring bar is encased in Teflon). When the powder is completely dissolved, cystine is added (see footnote, Table I) and the pH is adjusted (if required) by passing a mixture of 5 per cent CO_2 in air through the solution. The medium is sterilized by filtration (Selas filter of 02 porosity) and stored at 4° C. Glutamine and penicillin, because of their instability in solution, are added to the medium just prior to use (see footnote, Table I). If S103 is to be stored at 4° C. for prolonged periods, it may be desirable to omit the vitamins from the powder and add them later from a concentrated stock solution stored in the deep freeze. A concentrate (twofold or greater) of S103 is readily prepared from the powder. If this is done, it is necessary to omit calcium chloride from the powder. The size of the lot of powder is limited only by the capacity of the equipment available. For example, a ball mill jar of 1.3 gallon capacity which accommodates sufficient chemicals for 200 liters of S103 is currently being used.

All of the chemically defined media used for tissue culture studies in this laboratory over the past 4 years (more than 6,000 liters, including about 1,200 liters of S103) have been prepared in the manner described, without evidence of differences between samples from the same lot of powder. These include Tyrode's solution,[1] Earle's saline,[2] V-614,[3] 199,[4] 703,[5] 858,[6] MS,[7] S16, and S18,[8] Eagle's solution,[9] and many modifications of the above which are employed in studies on cell nutrition. Fat-soluble vitamins, ethanol, and Tween 80 are omitted from the powdered form of 199, 703, 858, and MS. It is emphasized that it may be expedient to omit additional components as dictated by the organization and needs of individual laboratories (see discussion of S103). The advantages of "certified" stocks of media are obvious in situations where it becomes necessary to trouble-shoot for the evasive "toxic factors" related to "water," "serum," or "glassware" which so frequently ambush the investigator.

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CHROMATOGRAPHY OF HEMOGLOBIN TYPES ON CARBOXYMETHYLCELLULOSE

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MUCH progress has been made, in recent years, in the field of ion exchange chromatography of proteins.[1] The ion exchange resin usually used in chromatographic studies of proteins is the carboxylic cation exchanger, Amberlite IRC 50. The chromatographic behavior of hemoglobin on this weak cation exchanger was first investigated by Boardman and Partridge[2, 3] who succeeded in separating some closely related adult and fetal animal hemoglobins. The same resin has been used for the chromatographic separation of different human hemoglobin types[4-6] and of some animal hemoglobins.[7-9]

In 1955, Peterson and Sober[10] and Sober and co-workers[11] developed some new adsorbents, derived from cellulose, which contained groups as carboxymethyl (CM), phosphate (P) and diethylaminoethyl (DEAE). These substances show a relatively great capacity for the adsorption of proteins, but elution can be performed under mild conditions. The use of some of these substances, especially carboxymethylcellulose (CMC), seems to offer possibilities for the separation of closely related hemoglobins, because chromatographic experiments performed by these investigators have indicated the presence of several components in some hemoglobin preparations.[12] In the present report we will describe the application of the CMC chromatography in detecting and quantitating different hemoglobin types. The chromatographic analysis has been applied to artificial and naturally occurring mixtures of different human hemoglobins (A, S, C, E, F, H, J, L, and Buginese-X[13] were available), as well as to some animal hemoglobins.

The present technique, while unsuitable for routine analyses, not only offered new possibilities for the characterization and quantitative estimation of different hemoglobin types, but also proved to be valuable for the preparation of pure hemoglobin components on a larger scale.

MATERIAL AND METHODS

Blood samples derived from patients suffering from various hemoglobinopathies and also from some animals were investigated. Next to samples of normal adult individuals (laboratory workers), the hemoglobins of the following abnormalities were studied: Hb-H disease, kindly supplied to us by Dr. Fessas (Athens, Greece) and sent by air under special precautions by which it was possible to investigate this sample 4 days after collecting

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the sample; Hb-J trait, obtained from members of a family described earlier[14]; 3 samples of cord blood from full-term infants and 1 from a 3-month-old premature infant; homozygous Hb-C disease, homozygous Hb-S disease (blood collected after the transfusion of blood from a normal adult), and the hemoglobin S-hemoglobin C disease, all sent to us by Dr. van der Sar (Curacao, Dutch West Indies); different samples containing Hb-E, both as the Hb-E trait and the Hb-E thalassemia disease, kindly supplied to us by Dr. de Silva (Ceylon) and obtained from members of a family described earlier[15]; 2 samples of thalassemia minor, 1 sent to us by Dr. Fessas (Athens, Greece) and 1 from a member of the family mentioned earlier[15]; 2 samples of thalassemia major, sent by Dr. de Silva (Ceylon); a sample of Hb-L trait, supplied by Dr. Lehmann (London, England); and a sample of the so-called Buginese-X hemoglobin,[13] sent by Dr. Lie Injo Luan Eng (Djakarta, Indonesia).

Blood samples of the following animals were also investigated: the adult cow and the fetal cow (derived from a 6-month-old cow fetus); the adult sheep, heterozygous for the 2 sheep hemoglobins I and II,[8] and a 3-week-old lamb possessing, next to the hemoglobin's I and II, the fetal component; the adult horse (a domestic stock); the hen (stock for slaughter); the Siamese jungle fowl or Bankiva; and the newborn chicken. The blood was obtained by venepuncture except for the blood from the fetal cow (by heart puncture) and from the fowl (by decapitation).

The erythrocytes were separated from the plasma by centrifugation, washed 3 times with normal saline, and hemolyzed with 1 volume of water and 0.5 volume of toluene. After standing overnight, the solution was centrifuged and finally cleared by filtration on Celite 535 or by centrifugation in the cold at 30,000 g for 30 minutes (as in the case of Hb-H and of the chicken).

The Chromatographic Procedure.—

The preparation of the cation-exchanger: Carboxymethylcellulose was prepared in the same way as described by Peterson and Sober[10] by reacting cellulose (Whatman cellulose powder passing through a 325 mesh sieve) in alkali with chloroacetic acid. After removal of the alkali in the manner described by the authors,[10] the final reaction product is equilibrated with a 0.01 M phosphate buffer of a special pH (6.8, 6.5, 6.3, or 6.0) by washing with the desired buffer several times. The clear supernatant was always used for the final determination of pH.

Preparation of the adsorbent column: The adsorbent was poured as a slurry into a column (55 by 0.9 cm.) and allowed to settle by gravity. After settling was complete, additional amounts of the slurry were introduced until (after settling) a height of 52 cm. was reached. The column was then mounted above a fraction collector and washed with buffer of the chosen pH for some hours to ensure equilibrium. Finally a column with a height of 50 cm. was used.

Development of the chromatogram: A small amount of a hemoglobin solution containing 100 to 150 mg. of hemoglobin was diluted to 5 ml. with the buffer solution of the chosen pH, converted to the monocarboxy form, and dialyzed for 24 hours against the same buffer at 4° C. This solution was placed on top of the column and allowed to penetrate into the adsorbent. Residual amounts were washed into the column with a 1 ml. portion of buffer before the continuous flow of buffer was begun. A gradient of pH was established by introducing a 0.01 M phosphate buffer solution pH 8.0 (prepared by adjusting the pH of 1,000 ml. of a solution containing 1.78 Gm. $Na_2HPO_4 \cdot 2H_2O$ per liter with about 35 ml. of a solution containing 1.56 Gm. NaH_2PO_4 per liter to a pH of 8.0) from a supply bottle into a constant volume (50 ml.) mixing chamber. In some cases a phosphate buffer of pH 7.5 was used. The mixing chamber and the tube between the mixer and the surface of the adsorbent, the volume of the latter being about 15 ml., contained the same buffer solution as used for the equilibration of both the adsorbent and the hemoglobin solutions. In general, this solution consisted of a 0.01 M phosphate buffer of pH 6.8, prepared in a similar way as given for the 0.01 M phosphate buffer of pH 8.0. In some cases, however, buffer solutions with lower pH's (6.5, 6.3, and 6.0) were required. In the case of Hb-C, replacement of the 0.01 M phosphate buffer of pH 8.0 in the supply bottle by a 0.01 M Na_2HPO_4 solution, after the elution of 100 ml. has been completed, was found necessary to obtain a sharp boundary

of this hemoglobin component. A flow rate of 4 to 6 ml. per hour was achieved under hydrostatic pressure by lowering or raising the supply bottle. In general a pressure of 30 to 50 cm. H_2O was found to be sufficient. The eluate was collected in 2 to 3 ml. fractions. All column operations were performed in a room with a temperature below 10° C. At the completion of the run, the CMC was regenerated with a mixture of equal volumes of 0.5 N NaOH and 0.5 M NaCl and subsequently washed with water to remove the alkali and sodium chloride.

Examination of the fractions: Each fraction was diluted with deionized water to 4 ml. and routinely examined in a Beckman DU spectrophotometer at 580 mμ. pH determinations (accuracy ± 0.01) were routinely performed at room temperature on the contents of every fifth tube (Polymetron SA, Zurich). Occasionally the pH determination was carried out in every fraction. Sometimes the contents of the tubes corresponding to one peak were combined and pervaporated to approximately 0.5 ml. A small amount of this fraction was then subjected to paper electrophoresis in veronal buffer (μ 0.05, pH 8.6) for 12 to 18 hours. The apparatus was the same as described earlier.[16]

Calculation of the percentual amounts of the hemoglobin components, by counting the extinctions of the relating fractions and dividing these values on the total extinction calculated, was possible since recovery experiments showed that over 97 per cent of the total hemoglobin added to the column was eluted.

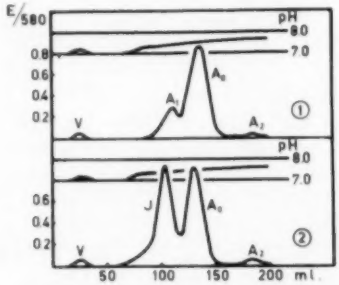


Fig. 1.—The separation of hemoglobins A and J on carboxymethylcellulose (gradient 6.8 to 8.0). 1 = Normal adult. 2 = Hb-J trait.

RESULTS

The Chromatographic Behavior of Normal Adult Hemoglobin on CMC.—Fig. 1 illustrates the elution diagram obtained when CO-hemoglobin of a normal adult was chromatographed. Four different components (called V, A_1, A_0, and A_2) were observed. Next to this elution diagram, the curve indicating the pH values of the different fractions is given. After an initial raise of the pH (from 6.9 to 7.2 at 25 ml.), the gradual increase of the pH is started at 60 to 70 ml. of the effluent. The existence of the first component of the elution diagram (V), varying between 0.5 and 2 per cent of the total amount of hemoglobin investigated, may be caused by the initial raise of the pH. The Hb-A_1 fraction, present for about 10 per cent, is eluted at a pH of 7.40 ± 0.01, the main fraction (Hb-A_0) at a pH of 7.49 ± 0.01, and the Hb-A_2 fraction, present in amounts varying between 0.5 and 2 per cent, at a pH of 7.69 ± 0.01. Paper electrophoretic analyses of these isolated fractions (Fig. 2) showed different electrophoretic mobilities in a veronal buffer ($\mu = 0.05$, pH 8.6). The Hb-A_1 moved slightly faster than the Hb-A_0, while Hb-A_2 possessed the same mobility as Hb-E. The possibility exists that these fractions of normal adult

hemoglobin are similar to those described by Kunkel and co-workers[17-18] using electrophoresis on starch slabs. These authors also observed 2 minor components in normal adult hemoglobin: one (Hb-A_2) being present for about 2 per cent and possessing an electrophoretic mobility similar to that of Hb-E, and a second with a mobility slightly higher than that of the main fraction.

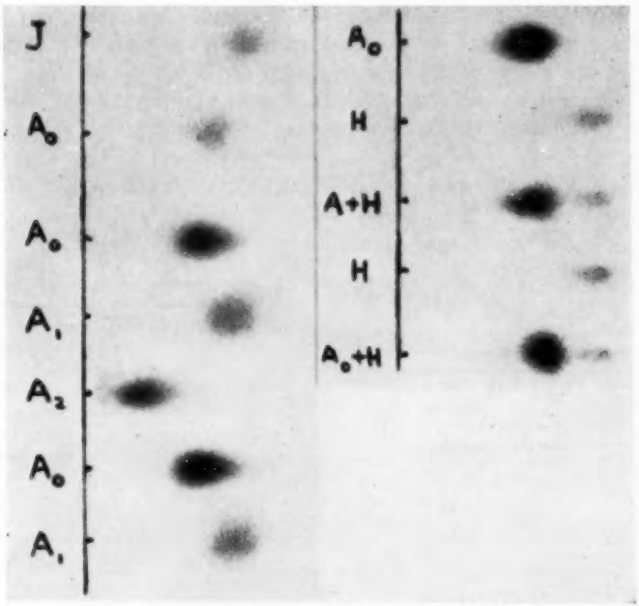


Fig. 2.—Paper electrophoresis of the isolated hemoglobin components A_0, A_1, A_2, J, and H.

A chromatogram of the hemoglobin derived from a patient with the Hb-J trait is also shown in Fig. 1. Next to the fractions V, A_0, and A_2, a high amount (over 50 per cent) of a component, which moved slightly faster in paper electrophoresis than the A_0 fraction (Fig. 2), was isolated. This Hb-J is indistinguishable from the Hb-A_1 both in paper electrophoresis (Fig. 2) and in CMC chromatography (Hb-J was eluted at a pH of 7.39 ± 0.01 and Hb-A_1 at a pH of 7.40 ± 0.01).

The Chromatographic Behavior of Hemoglobin H on CMC.—Fig. 3 illustrates the results of the chromatographic behavior of the hemoglobin derived from a patient suffering from the Hb-H disease. Using the gradient system pH 6.8 to pH 8.0 the Hb-H moved quickly and was completely eluted within 40 ml. The elution curve showed 2 fractions of Hb-H; the appearance of these two peaks correlated with the temporate increase of the pH (from 6.9 to 7.3) and of the sodium ion concentration of the effluent. Next to the Hb-H, the 3 fractions of Hb-A (A_1, A_0, and A_2) were found to be present. The elution rate of Hb-H can be decreased by lowering the pH of the initial elution buffer. With the gradient system pH 6.5 to pH 8.0, over 90 per cent of the Hb-H was

eluted within 50 ml. Again 2 fractions appeared which correlated with a temporate raise of the pH from 6.6 to 7.0. A small amount of Hb-H remained at the top of the column and was eluted as soon as the gradient developed. Lowering the pH of the initial elution buffer to 6.0 (gradient system pH 6.0 to pH 8.0) caused an almost complete elution of Hb-H at the time the gradual increase of the pH reached a pH value of 6.7 to 6.9. The initial increase of the pH (from 6.2 to 6.6) is accompanied by the elution of only a small amount of the abnormal component. The identity of the different fractions was studied by paper electrophoresis. Fig. 2 illustrates some of these results, indicating the isolation of pure Hb-H by this chromatographic method. In conclusion, it is evident that the abnormal Hb-H can be obtained as one single boundary as soon as the gradual increase of the pH of the effluent reaches the value of about 6.8, while the initial pH of the elution buffer must remain below a value of 6.5 The amount of Hb-H present in the sample was about 15 per cent of the total hemoglobin content.

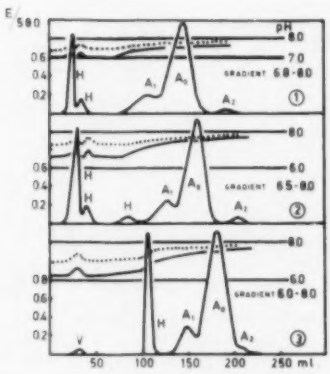


Fig. 3.

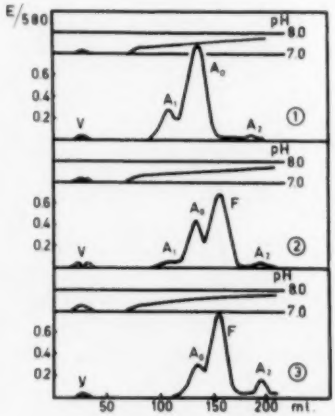


Fig. 4.

Fig. 3.—The separation of Hb-H on carboxymethylcellulose with different pH gradients. (The dotted lines indicate the concentration of sodium in the effluent.)

Fig. 4.—The separation of adult and fetal hemoglobin on carboxymethylcellulose (gradient 6.8 to 8.0). 1 = Normal adult. 2 = Full-term infant. 3 = Three-month-old premature infant.

The Separation of Adult and Fetal Human Hemoglobin.—Two examples of the separation of fetal and adult hemoglobin present in hemoglobin samples of cord blood are given in Fig. 4. The fetal component was eluted at a pH of 7.58 ± 0.01 and could easily be distinguished from the 3 adult components. In the first sample of cord blood (full-term infant) all 3 adult components were present. The Hb-A_1 fraction was not detectable in a sample of blood derived from a 3-month-old premature infant, while the percentual amount of Hb-A_2 was increased several times in this sample (Fig. 4, 3).

Studies were also performed to compare the quantitative estimation of the fetal hemoglobin using the CMC chromatography and an alkali denaturation

technique. Fig. 5 shows the results of these investigations; the alkali denaturation method used was the spectrophotometric procedure described by Jonxis and Visser.[19] In general, a good agreement exists; however, it was found that the chromatographic procedure is unsuitable for the estimation of low percentages of fetal hemoglobin (below 5 per cent).

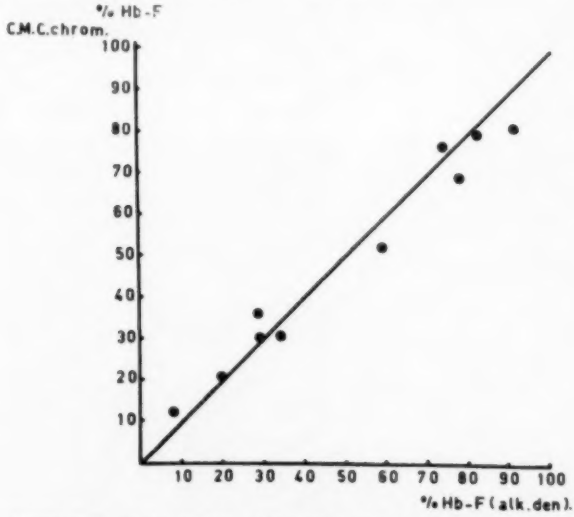


Fig. 5.—Comparison of the results of the estimation of Hb-F by chromatography on carboxymethylcellulose and by an alkali denaturation technique.

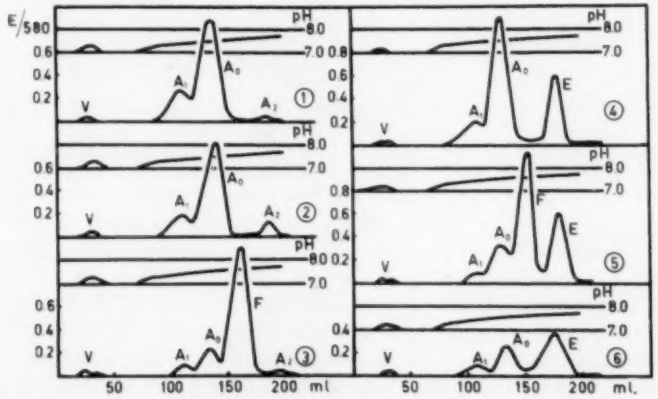


Fig. 6.—The separation of the hemoglobins A, A_2, F, and E on carboxymethylcellulose (gradient 6.8 to 8.0). 1 = Normal adult. 2 = Cooley's trait. 3 = Cooley's anemia. 4 = Hemoglobin-E trait. 5 = Thalassemia Hb-E disease. 6 = A case with a high amount of Hb-E.

Chromatographic Studies of the Hemoglobin in Thalassemia, Hemoglobin E Trait, and Hemoglobin E-Thalassemia Disease.—Fig. 6 illustrates the separation of the different hemoglobin components in the thalassemia syndrome and in

cases in which Hb-E is concerned. The hemoglobin in a case of thalassemia minor (Fig. 6, 2) showed a normal picture, except for an increased amount of the Hb-A_2 fraction (5 per cent). It will be noted that no fetal hemoglobin was present. The hemoglobin of a patient suffering from thalassemia major (Fig. 6, 3) consisted of a high amount of fetal hemoglobin (77 per cent by chromatography and 69 per cent by alkali denaturation) and the normal adult components A_1, A_0, and A_2. The amount of A_2 in this blood sample was about 1 per cent. In cases of heterozygous Hb-E disease (Fig. 6, 4) and thalassemia Hb-E disease (Fig. 6, 5), a good separation was obtained between Hb-E, Hb-F, and the 2 normal adult fractions, A_1 and A_0. No differences were found between Hb-A_2 and Hb-E, the former being eluted at a pH of 7.69 ± 0.01 and the latter at a pH of 7.68 ± 0.01. Finally, a case is presented (Fig. 6, 6) in which, next to a high amount of Hb-E (55 per cent), the 2 adult fractions A and A_0 were present, but no fetal hemoglobin was found.

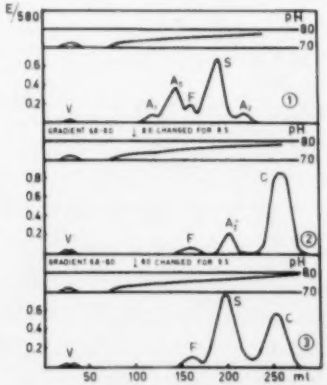


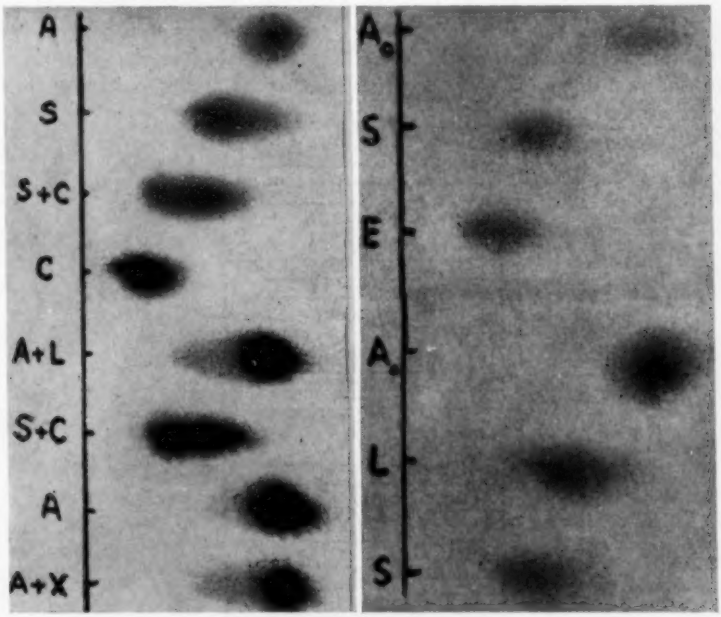
Fig. 7.—The separation of hemoglobins S and C on carboxymethylcellulose. 1 = Homozygous Hb-S (blood obtained after blood transfusion). 2 = Homozygous Hb-C. 3 = Hemoglobin S-hemoglobin C disease.

Chromatographic Behavior of Hemoglobins S and C.—Fig. 7 shows the chromatographic elution curves obtained for cases of homozygous Hb-S disease (blood taken after blood transfusion), homozygous Hb-C disease, and hemoglobin S–hemoglobin C disease. The paper electrophoretic pattern of these cases is presented in Fig. 8, *A*. In the blood of the patient with sickle-cell anemia, next to Hb-S, the 3 normal adult hemoglobins (A_1, A_0, and A_2) and also the fetal component were present. The identification of Hb-F was confirmed by its specific ultraviolet spectral absorption. The presence of the adult components may be explained by the transfusion of normal adult blood. It is remarkable, however, that the amount of Hb-A_2 is increased (5.5 per cent). The isolated Hb-S (eluted at a pH of 7.64 ± 0.02) showed one boundary in paper electrophoresis (Fig. 8, *B*).

The isolation of Hb-C (Fig. 7, 2) required the application of a phosphate solution of pH 8.5 in the supply bottle. This solution was introduced after the elution of about 100 ml. Next to Hb-C (eluted at pH 7.82 ± 0.02), a low

amount of fetal hemoglobin (3.5 per cent) was present; the identification of this component was confirmed by ultraviolet spectral absorption measurements. Remarkable was the high amount (8.5 per cent) of a fraction eluted at the same pH (7.70) as found for the Hb-A_2 fraction (7.69 ± 0.01). The possibility exists that these 2 fractions are identical. No Hb-A_1 or Hb-A_0 was present.

Finally, in the case hemoglobin S–hemoglobin C disease, a complete separation of these 2 components was obtained. No normal adult components were found, but a small amount of the fetal hemoglobin (5.5 per cent) was again demonstrated.



A. *B.*

Fig. 8.—*A*, Paper electrophoretic pattern of the blood samples of some hemoglobinopathies. *B*, Paper electrophoretic pattern of some isolated hemoglobin types.

The Identification of the Hemoglobins L and Buginese-X.—Fig. 8 shows some studies on the chromatographic behavior of 2 recently discovered hemoglobins, L[20] and Buginese-X,[13] both migrating between the normal adult and sickle-cell hemoglobin in paper electrophoresis (Fig. 8, *A*). The Hb-L was eluted in CMC chromatography at pH 7.71 and was nearly indistinguishable from Hb-A_2 and Hb-E by this method. The separation of Hb-L from the adult components A_1 and A_0 was complete. The Hb-L is different from Hb-S since, after addition of pure sickle-cell hemoglobin (prepared by elution using the same CMC chromatography), a separation of Hb-L and Hb-S, as well as hemoglobins A_1 and A_0, was obtained (Fig. 9, 2). Fig. 8, *B*, represents the

paper electrophoretic patterns of isolated Hb-L. Hb-X, on the contrary, was eluted at a much lower pH (pH 7.61) than was found for Hb-L (Fig. 9, 3). Next to the Hb-X, Hb-A_0, and Hb-A_1 fractions, a small amount of Hb-A_2 was eluted also. As Hb-X showed about the same behavior in CMC as that found for Hb-S, this hemoglobin (together with Hb-C) was added. An incomplete but clearly visible separation of Hb-X and Hb-S was obtained (Fig. 9, 4) indicating a different behavior of these two hemoglobins in CMC chromatography. Finally, addition of the isolated pure Hb-L to the Buginese-X hemoglobin offered further proof for the difference of these two abnormal hemoglobins (Fig. 9, 5). From these comparative studies, it is evident that the two hemoglobins L and X are different components, confirming analytical data obtained by Lehmann in paper electrophoresis[21] and chromatographic differences on Amberlite IRC 50.[22] Since the hemoglobin Buginese-X is also different from Hb-G,[21] the possibility exists that this hemoglobin is a newly discovered abnormal component.

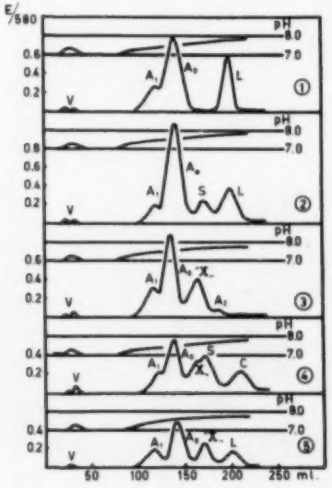


Fig. 9.—The separation of hemoglobins L and Buginese-X on carboxymethylcellulose (gradient 6.8 to 8.0). 1 = A + L. 2 = A + L with addition of S. 3 = A + X. 4 = A + X with addition of S + C. 5 = A + X + A + L.

Quantitative Results.—Table I summarizes the percentual amounts of the components observed in normal and pathologic conditions. In blood specimens obtained from 7 normal adult individuals (laboratory workers) about 10 per cent of Hb-A_1 and about 1 per cent of Hb-A_2 were found to be present, next to small amounts of the first component V (the identity of which is still unknown) and high amounts of the main component Hb-A_0. The percentages of Hb-A_1 vary between 8 and about 16 per cent and of Hb-A_2 between 0.5 and 2 per cent. These values are in reasonable agreement with the data obtained by Kunkel and associates[18] using starch block electrophoresis. In 3 cases of blood obtained from the umbilical cord of newborn infants the

presence, next to high amounts of Hb-F, of the 3 adult hemoglobin components was established. The percentual amount of Hb-A_2 fraction was slightly lower than found in the normal group. In a blood specimen obtained from the umbilical cord of a 3-month-old premature infant, however, a high percentage (6 per cent) of Hb-A_2 was present. In blood samples from 2 individuals with thalassemia trait also, increased amounts of Hb-A_2 were obtained, which agrees with the results of electrophoretic studies on starch slabs[18] and on paper.[23, 24] It will be noted that in the 2 blood samples no fetal hemoglobin was detectable. Blood samples from 2 patients with Cooley's anemia were also studied. Next to high amounts of Hb-F, no increased percentage of Hb-A_2 was observed.

TABLE I. THE AMOUNTS OF DIFFERENT HEMOGLOBIN COMPONENTS IN NORMAL ADULTS, CORD BLOOD SAMPLES, COOLEY'S TRAIT AND ANEMIA CASES, HETEROZYGOUS AND HOMOZYGOUS HB-E DISEASE, AND IN SOME CASES WITH THE HB-E THALASSEMIA DISEASE

CASE	V	A_1	A_0	F	A_2	E
Adult (7 cases)	1.5 (1-2)	10.5 (8-15.5)	87 (84-90.5)	—	1 (0.5-2)	—
Cord blood	0	0	20	79.5	0.5	—
Cord blood	1.5	3.5	25.5	69	0.5	—
Cord blood	1	2.5	19.5	76	1	—
Cord blood (prem.)	2	0	11	81	6	—
Cooley's trait	1.5	12	81.5	0	5	—
Cooley's trait	1	6.5	86.5	0	6	—
Cooley's anemia	1	4	17	77	1	—
Cooley's anemia	1	12.5	55.5	30.5	0.5	—
Hb-E trait	0.5	8.5	62.5	—	—	28.5
Hb-E trait	2.5	10	71.5	—	—	16
Hb-E trait	1	11	62	—	—	26
Hb-E trait	0.5	9.5	70.5	—	—	19.5
Hb-E thalass.	0	6.5	30	30	—	33.5
Hb-E thalass.	1.5	0	15	35.5	—	48
Hb-E thalass.	1	3.5	10	52.5	—	32.5

Considerable variation in the amounts of Hb-E was found to be present in 4 cases of the heterozygous Hb-E disease (15.5 to 28.5). It is remarkable that the percentages of Hb-E hardly exceeded 25 per cent of the total hemoglobin. Moreover, low percentages of Hb-E (15.5 per cent, for instance) make a differentiation between the Hb-E trait and Cooley's trait, with a high amount of the Hb-A_2, almost impossible. Clinical, hematologic, and genetic studies are of great importance for a definite recognition of these hemoglobin abnormalities. Finally, blood specimens from 3 cases with the double heterozygous condition, Hb-E thalassemia, were investigated. A calculation of the percentual amount of the different fractions A_1, A_0, F, and E was easily possible. The Hb-E content, as well as the Hb-F content, varied between 30 and 50 per cent in our cases. It will be noted that a reasonably constant ratio is present in these different cases, between the amounts of Hb-A_1 and Hb-A_0, the latter being present in amounts about 5 to 8 times higher than the Hb-A_1 fraction.

Table II summarizes the quantitative data obtained in some single cases of hemoglobinopathies. Hb-H was present in low amounts (15 per cent), as also was Hb-X (18.5 per cent) and Hb-L (25 per cent). The Hb-J trait, on the contrary, possessed high amounts of the abnormal fraction (over 50 per cent). Small amounts of fetal hemoglobin (3.5 to 7.5 per cent) were found

in some cases, in which Hb-S and/or Hb-C were concerned. Attention will be drawn also to the high amounts of the Hb-A_2 fraction in some of these cases.

TABLE II. THE PERCENTUAL AMOUNTS OF DIFFERENT HEMOGLOBIN COMPONENTS IN SOME HEMOGLOBINOPATHIES

CASE	V	A_1	A_0	F	A_2	ABNORMAL COMPONENT
Normal adult (7 cases)	1.5 (1-2)	10.5 (8-15.5)	87 (84-90.5)	—	1 (0.5-2)	—
Hb-H disease	—	11	73	0	1	Hb-H : 15 %
Hb-J trait	1.0	?	45.5	0	2	Hb-J : 51.5%
Buginese-X trait	1.5	11	67.5	—	1.5	Hb-X : 18.5%
Hb-L trait	0.5	9.5	65	0	?	Hb-L : 25 %
Sickle-cell anemia (after blood transfusion)	0.5	4.5	21	7.5	5.5	Hb-S : 61 %
Hom. Hb-C disease	0.5	0	0	3.5	8.5	Hb-C : 87.5%
Hb-S/Hb-C disease	0.5	0	0	5.5	?	Hb-S : 49.5% Hb-C : 44.5%

The Chromatographic Behavior of Partially Oxidized Carbonmonoxy-Hemoglobin.—Recently Itano and Robinson[25] described the separation of intermediate forms of carbonmonoxy- and ferrihemoglobin by the moving boundary electrophoresis, providing the first conclusive demonstration of their existence.

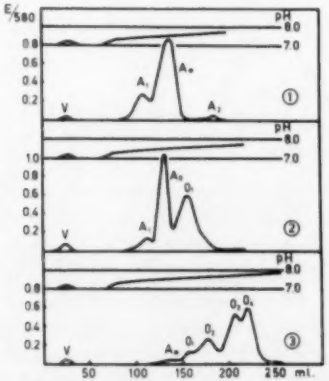


Fig. 10.—The separation of partially oxidized hemoglobin derivatives on carboxymethylcellulose (gradient 6.8 to 8.0). 1 = Normal adult. 2 = Normal adult; 1,000 mg. COHb in 0.01 M phosphate buffer (pH 6.8) oxidized with 10 mg. $K_3Fe(CN)_6$ for 2 hours. After dialysis for 48 hours against the same buffer, 100 mg. of hemoglobin is chromatographed. 3 = Normal adult (as in 2) but oxidation was carried out with 40 mg. $K_3Fe(CN)_6$.

Studying the separation of these intermediate components by the CMC chromatography, it was found that this technique offers new possibilities in preparing larger quantities of these derivates. The oxidation of the normal adult hemoglobin was carried out in a similar way as described by Itano and Robinson[25] using $K_3Fe(CN)_6$ in amounts equivalent to 50 per cent and 200 per cent of hemes present. The final dialysis was carried out against the 0.01 M phosphate buffer pH 6.8 necessary in the CMC chromatography. The results of the chromatographic separation are given in Fig. 10. In the first experiment

(Fig. 10, 2), in which 50 per cent of $K_3Fe(CN)_6$ was added to the COHb-A, 3 different peaks were observed. The first 2 were identical with the normal adult components A_1 (5 per cent) and A_0 (42 per cent), while the third (52 per cent) was eluted at a pH of 7.56. Addition of a twofold excess of $K_3Fe(CN)_6$ to the COHb-A resulted into the presence of 5 different components. A small amount of the A_0 component was still present and next to this 4 different intermediate forms (O_1, O_2, O_3, and O_4) were observed, which were eluted at pH's 7.58, 7.63, 7.70, and 7.75, respectively. The spectral absorption of these different components is given in Fig. 11. The transmission curves of the O_1, O_2, O_3, and O_4 fractions were quite different from that of COHb-A_0 and of ferrihemoglobin. All showed, next to the Soret maximum (410 to 420 mμ), 4 different maxima at 490, 540, 580, and 630 mμ. Two of these are specific for the carbonmonoxy form (540 and 580 mμ), and 2 are specific for the ferrihemoglobin (490 and 630 mμ). It is remarkable, however, that the heights of these maxima increase to high values.

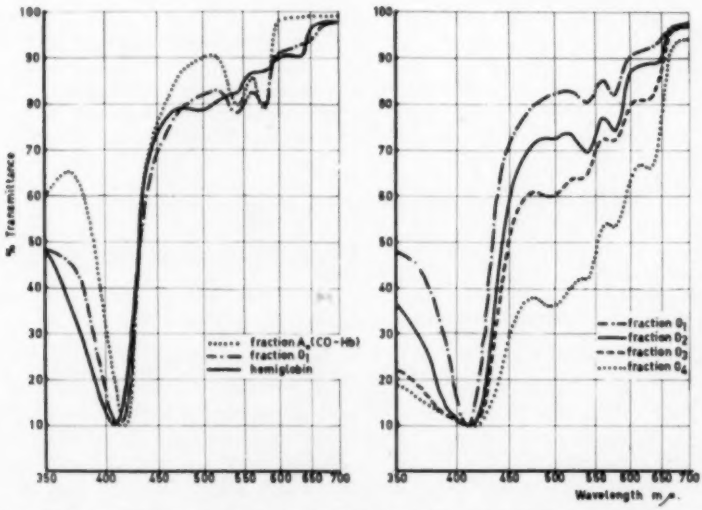


Fig. 11.—The transmission curves of the isolated partially oxidized hemoglobin fractions. The symbols correspond with those given in Fig. 10.

The Application of the CMC Chromatography for the Study of Animal Hemoglobins.—Fig. 12 illustrates some separations of animal hemoglobins. In the blood samples of a few horses (domestic stock), 2 different components were observed, which also could be separated in the moving boundary electrophoresis. The first peak (present for 58 per cent) was eluted at a pH 7.35 and the second (40 per cent) at a pH of 7.47. In a lamb, possessing next to fetal hemoglobin the 2 adult hemoglobins I and II,[8] 3 different fractions were observed: the Hb-F being eluted at a pH of 7.15, the Hb-II at a pH of 7.23, and the Hb-I at a pH of 7.32 (Fig. 12, 2). In this experiment a gradient with 2 phosphate buffers with a pH of 6.3 and 7.5 was found necessary. The fetal

and adult hemoglobin of the cow could be separated very easily using the gradient system pH 6.8 to 8.0. In Fig. 12, 3, an example of the separation of these 2 components (artificial mixture) is given. The fetal hemoglobin moved very quickly while the Hb-A of the cow was eluted at a pH of 7.40. The pattern obtained in studying the hemoglobin of the hen (a domestic stock as well as the jungle fowl) and of the newborn chicken were completely similar. A separation of 2 mean fractions was obtained, the presence of which was demonstrated earlier.[7]

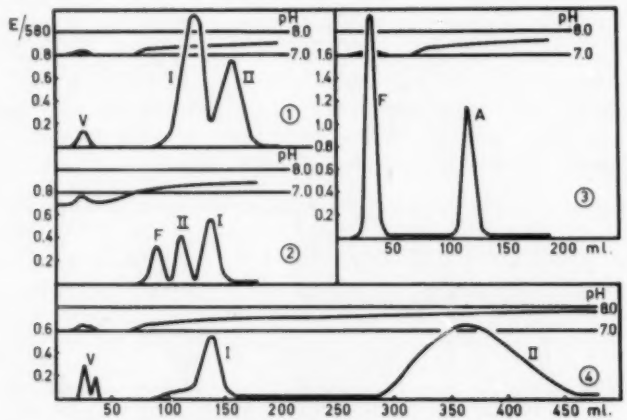


Fig. 12.—The separation of some animal hemoglobins on carboxymethylcellulose. 1 = Adult horse. 2 = Lamb possessing next to Hb-F, the hemoglobins I and II. 3 = Mixture of hemoglobins A and F of the cow. 4 = Adult chicken.

DISCUSSION

The CMC chromatographic technique has been employed for the detection and separation of many human hemoglobin components and of some animal hemoglobin types. In general, a good and sometimes complete separation of 2 or more different components was obtained. It offers not only new possibilities for the identification of hemoglobin components, but it also makes quantitation of hemoglobin types in mixtures and the preparation of larger quantities of pure hemoglobin components possible. A great disadvantage of the technique, however, is that the method is not suitable for routine analyses.

The present experiments on the hemoglobin of normal adults showed the existence of at least 3 fractions (A_1, A_0, and A_2), offering further evidence of the heterogeneity of normal adult hemoglobin.[26] The paper electrophoretic studies of the isolated fraction made their identity with the 3 fractions obtained by Kunkel and Wallenius,[17] using starch block electrophoresis, likely. Several attempts have been made to detect the nature of the fourth compound present in the hemoglobin of normal adults, as well as in many other hemoglobin samples, which is eluted at a low pH and found in low quantities. Sometimes this component was resolved into 2 fractions. Although the initial increase of pH may cause the elution of a small amount of the hemoglobin,

which is attached to the adsorbent, other explanations are still possible. It was found that addition of small quantities of uncolored proteins (serum proteins, for instance) increased the amount of this so-called fraction V, indicating that this fraction may consist of hemoglobin combined with some (unknown) protein. Careful spectral studies, however, in visible as well as in ultraviolet light did not offer strong evidence for a suchlike combination, especially not for the second fraction, when this component is resolved into 2 peaks. Another possibility, namely, the existence of an unknown component (perhaps Hb-H?) present in small amounts in the different blood samples, still exists. Great difficulty, however, is encountered in working with this fraction because of the ease of denaturation.

TABLE III. A COMPARISON OF THE BEHAVIOR OF DIFFERENT HUMAN HEMOGLOBIN TYPES ON CARBOXYMETHYLCELLULOSE WITH SOME OTHER METHODS

HB-TYPE	N*	ELUTION pH	RELATIVE MOBILITIES		ISOELECTRIC POINTS CO-HB
			IN PAPER ELECTROPHORESIS (pH 8.6)	IN CHROMATOGRAPHY ON AMBERLITE IRC 50	
Hb-H	4	6.7 – 6.9	13	15	5.6
Hb-J	8	7.39 ± 0.01	12	10	—
Hb-A_1	29	7.40 ± 0.01	—	—	—
Hb-A_0	40	7.49 ± 0.01	10	10	6.87
Hb-F	18	7.58 ± 0.01	9-10	13.5	6.98
Hb-X	3	7.61	9	10	—
Hb-S	7	7.64 ± 0.02	7	7.5	7.09
Hb-E	13	7.68 ± 0.01	5	9	7.20
Hb-A_2	15	7.69 ± 0.01	5	—	—
Hb-L	3	7.71	7-8	5.5	—
Hb-C	6	7.82 ± 0.02	4	3.5	7.39

*N = number of estimations.

A comparison of the behavior of the different human hemoglobin components on CMC chromatography with their electrophoretic pattern and their behavior on Amberlite IRC 50[5, 6] is given in Table III. A general relation was found to exist between the "elution pH values" of the different human hemoglobin types and their relative mobilities in paper electrophoresis at pH 8.6. Hemoglobin C has the lowest electrophoretic mobility and is eluted at the highest pH value, while the 2 "fast-moving" hemoglobins studied (H and J) are eluted before the main fraction of normal adult hemoglobin. This relation may indicate that the separation of the hemoglobin types in CMC chromatography is mainly based on differences in charge of the hemoglobin molecule. Only one exception exists: the abnormal Hb-L. This hemoglobin type behaves on CMC in a similar way as found in Amberlite IRC-50 chromatography; Hb-L is eluted after Hb-S and before Hb-C. A similar relation between chromatographic behavior and electrophoretic mobility (in the moving boundary electrophoresis) was also found to exist for the intermediate forms of Hb-A formed after partial oxidation of this hemoglobin with $K_3Fe(CN)_6$.

Studying the elution pH values more closely, it is clear that no differences exist between those of the Hb-J and Hb-A_1 fraction (7.39 and 7.40, respectively). Therefore, the possibility exists that these 2 hemoglobin components are identical, in consequence of which the hemoglobinopathy Hb-J trait must

be regarded as an abnormality in the production of the Hb-A_1 fraction. The elution pH values for Hb-E and Hb-A_2 fraction (7.68 and 7.69, respectively) were also found to be the same.

The behavior of Hb-H is well defined in regard to the other human hemoglobin types since this hemoglobin is eluted at the low pH value of about 6.8. Therefore, this technique offers a good method for the preparation of large quantities of pure Hb-H. The marked chromatographic as well as electrophoretic behavior of Hb-H may be related to structural differences between this hemoglobin type and the normal adult hemoglobin because the estimation of the amino acid composition revealed, among other things, a higher amount of glutamic acid and a corresponding lower amount of alanine (unpublished results).

The chromatographic separation of Hb-F offers a new means for the estimation of this hemoglobin in blood samples. For cord blood samples, as well as for blood samples of patients suffering from thalassemia major and thalassemia Hb-E disease, a good relation exists between the values obtained with this new chromatographic procedure and with the alkali denaturation technique. Moreover, some examples are given of the estimation and identification of low quantities of Hb-F in some cases of sickle-cell disease and homozygous Hb-C disease.

The chromatographic behavior of Buginese-X hemoglobin is an example of the possibilities offered by this method for the identification of abnormal components. In the case of Buginese-X, a definite difference between this hemoglobin and the hemoglobin types A_0, F, S, and L was demonstrated.

The few quantitative results may raise questions, but the number of analyses given in Tables II and III is too small to permit any conclusions. Further research will be necessary.

The results of analyses of the blood samples of some animals (horse, sheep, cow and hen) are given only as examples of the new procedures available for the study of hemoglobins in different species.

SUMMARY

A new chromatographic procedure is described using carboxymethylcellulose as cation exchanger and dilute phosphate buffers of low ionic strength with pH values between 6.0 and 8.5 as eluting solvents. With this technique, next to the normal adult and fetal human hemoglobins, 7 abnormal hemoglobin types (H, J, Buginese-X, S, E, L, and C) and the hemoglobins of some animals were investigated. In most cases a good and sometimes complete separation was obtained, offering a new method for the identification of the abnormal component. As a result of this procedure, the identity of the Buginese-X hemoglobin with Hb-S and Hb-L could be excluded. The pH values at which the different components were eluted are related to their relative mobilities on paper electrophoresis at pH 8.6, with the exception of Hb-L. This hemoglobin type showed the same behavior in CMC chromatography as found in Amberlite IRC-50 chromatography. Special attention was

drawn to the behavior of Hb-H, which was eluted at a relatively low pH value. New possibilities are presented for the quantitative estimation of the fetal hemoglobin and the detection of this component in some hemoglobinopathies.

The heterogeneity of the hemoglobin of normal adults, as demonstrated by different techniques, was found also using this new method. The existence of 3 different fractions (A_1 with the same behavior as Hb-J, A_0, and A_2 with the same behavior as Hb-E) was confirmed.

It was found possible to quantitate the amounts of the different components in mixtures. However, the small number of quantitative analyses did not allow us to draw definite conclusions.

Finally, examples of the separation of different hemoglobin components present in some animals are given.

We are highly indebted to Drs. Fessas, Lehmann, Lie Injo Luan Eng, van der Sar, and de Silva for sending the different blood samples.

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STUDIES OF RHEUMATOID SERUM EMPLOYING A MODIFIED COOMBS' SLIDE TEST

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BOSTON, MASS.

SINCE the initial observation by Waaler[1] of the enhanced agglutination of sensitized sheep erythrocytes by serum of patients with rheumatoid arthritis, a concerted effort has been made to determine the value of this phenomenon as a diagnostic test. The chemical nature and basic significance of "rheumatoid factor" are also being intensively investigated. The results of these studies have recently been summarized.[2-4] Rheumatoid factor activity has been shown by numerous investigators to reside in the globulin fraction of serum.[5-7] Studies by Franklin and associates[8] strongly suggest that rheumatoid factor is a macroglobulin but raise some question as to whether the same factor that combines with human gamma globulin is responsible for the agglutination of sensitized sheep red cells.

Numerous studies have indicated that the sheep erythrocytes used in the early tests for rheumatoid factor are not an essential part of the test system, and that any suitably sized particles which can be coated with gamma globulin can be agglutinated in the presence of rheumatoid factor. Thus, human red cells coated with antihuman red cell antibodies or Rh system antibodies[9-11]; sheep red cells treated with tannic acid and coated with human fraction II[12]; latex particles coated with gamma globulin[13, 14] and bentonite coated with gamma globulin[15] have all been shown to be agglutinated by some factor found mainly in the serum of patients with rheumatoid arthritis. The observation of Ziff and associates[16] that rheumatoid factor activity which is found in the euglobulin fraction of serum is inhibited by something in the euglobulin fraction of normal serum also suggested that rheumatoid factor combines with some component of normal serum globulin. These observations culminated in the report of Epstein and co-workers[17] of a precipitin reaction occurring between rheumatoid factor and gamma globulin.

In the course of evaluating patients with a variety of joint afflictions with a number of the already existing tests for rheumatoid factor activity we were stimulated to develop still another test by what appeared from our own experience to be some of the disadvantages of available methods. It is the main purpose of this report to describe this test, compare our results with it to our results with prior methods and to point out the utility of this test in both clinical and investigative work.

From The Arthritis Research Laboratory of The Boston Dispensary, Boston, Mass.
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Received for publication Feb. 17, 1958.

METHODS AND MATERIALS

Blood specimens were obtained mainly from inpatients at the New England Center Hospital and patients of the Arthritis and Medical Clinics of the Boston Dispensary. Sera to be tested were collected weekly and stored in the frozen state until used. Before use the sera were heated at 56° C. for 30 minutes in a water bath to inactivate complement. They were then treated by shaking with equal volumes of washed unsensitized sheep red blood cells to remove heterophil agglutinin.

The sensitized sheep cell agglutination test (S.S.C.A.) was performed by the method of Alexander and DeForest.[18] Fresh sheep red cells preserved in Alsever's solution were obtained as needed from the Hyland Laboratory. They were washed 3 times in physiologic saline and stored in the desired concentrations for not more than 48 hours before use. Rabbit antisheep cell serum obtained from the Markham Laboratory was made up as a stock solution of 1:100 in saline. The sensitizing hemolysin concentration was one-fifth the minimal agglutinating titer. With this method, titers of 1:64 or higher are considered positive.

Euglobulin fractions of serum were prepared by the method of Ziff and associates and used instead of whole serum in the method of Alexander and DeForest. The euglobulin inhibition test was not carried out. Although this method has considerable diagnostic accuracy, we feel that it is too lengthy and involved for routine clinical use.

The latex-fixation test was performed by the Rheins and co-workers' "drop" modification[19] of the test of Singer and Plotz. The latex particles used were supplied by the Dow Chemical Corporation. The gamma globulin used in this test, the slide test to be described, and in the inhibition studies was either poliomyelitis immune globulin* or a preparation of lyophilized fraction II kindly supplied by the Harvard Blood Fractionation Laboratory. All dilutions of gamma globulin were made in the physiologic saline.

The Rheins' method requires small amounts of serum and inactivation and absorption of the serum are unnecessary. The tests can be set up and read within an hour once the "antigen" has been prepared. In our experience the chief disadvantage of this method is that the strength of agglutination in the "positive" tests varies, making reliably reproducible readings difficult. Despite this disadvantage, we have had only one discrepant result in comparing this test to our slide test and the S.S.C.A. test on 35 sera and euglobulin fractions.

The Modified Coombs' Slide Test.—In its ability to agglutinate red cells coated with human gamma globulin, rheumatoid factor is remarkably similar to Coombs' antihuman globulin serum, which is ordinarily produced by immunizing rabbits against human gamma globulin. By taking advantage of the recently described ability of certain metallic cations in proper concentration to bind proteins to the surface of cells,[20] we found it possible to render sheep or human red cells Coombs' positive by shaking a suspension of cells in a mixture of gamma globulin and chromic chloride. For most batches of cells, a solution of chromic chloride (in saline) containing 4 to 5 μm per milliliter was satisfactory. The fact that the cells are properly coated is ascertained by doing a slide test with commercial Coombs' serum (Ortho). The Coombs' test on cells coated in this manner is 2 to 3 plus. Once the cells have been properly coated, they are used in performing Coombs' slide tests, but the patient's serum or euglobulin fraction is used in the place of Coombs' serum. The coated cells can be stored in the refrigerator and continue to give consistent results for at least one week. The reaction is easily read over a light in one minute. Sheep red cells or human type O red cells may be used interchangeably. If human cells are used, preliminary absorption and inactivation of the sera to be tested are unnecessary.† If only occasional tests are done, the patient's whole blood may be collected, and his own red cells may be separated and coated and used in the test.

*Manufactured by Cutter Laboratories, Berkeley, Calif.

†Slide tests against coated human type O cells were done with 20 sera which had not been inactivated or absorbed. The tests were repeated after inactivation at 56° C. for 30 minutes and absorption with two volumes of packed sheep red cells. There was no change in results. As a control, slide tests can be done simultaneously against washed, uncoated type O cells, but this does not appear to be necessary.

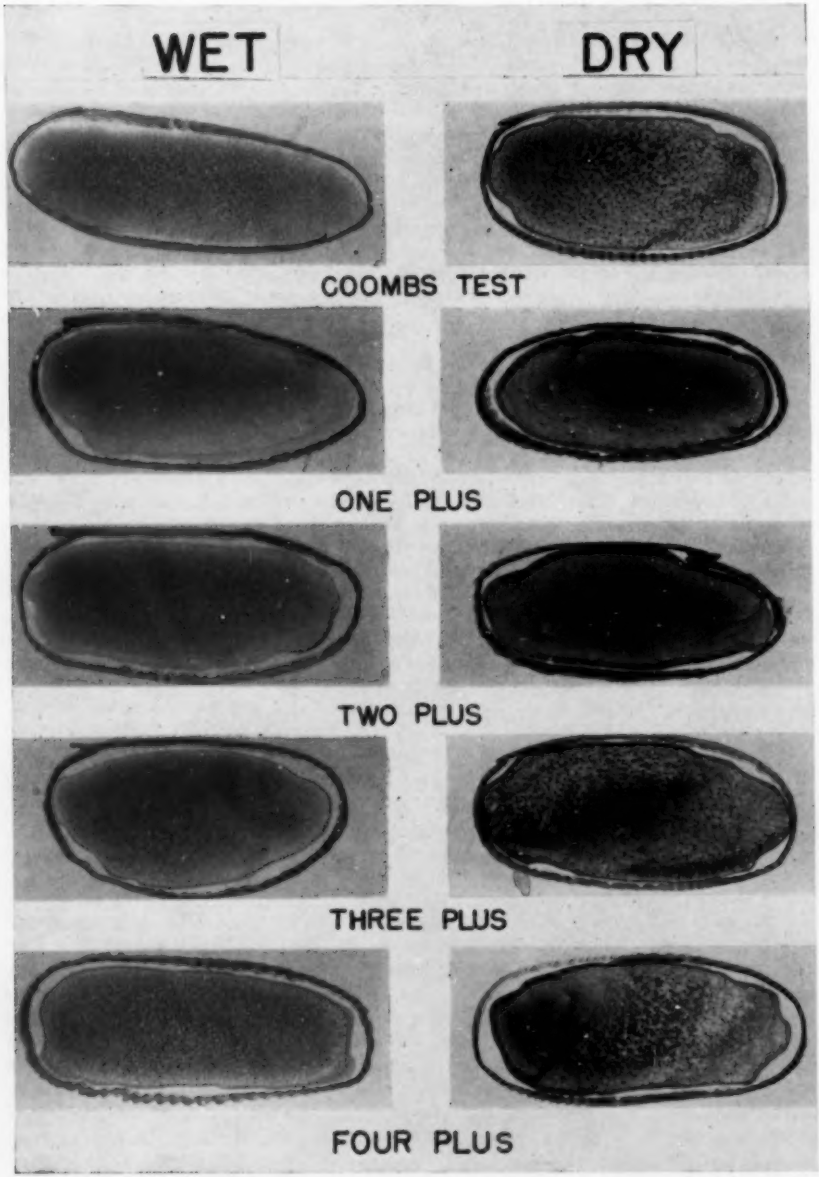

Fig. 1.—Reading from top to bottom, the first slide in each column is the result of a Coombs' test on the coated cells using commercial Coombs' serum. The remainder are the results of testing various rheumatoid sera against coated cells. Reactions designated as one plus are easily read by tilting the slide back and forth over a light source. They do not photograph well.

The exact details of the test are as follows: 0.5 ml. of 50 per cent washed red cells are suspended in 2.5 ml. of 1 per cent gamma globulin solution. One milliliter of chromic chloride solution containing 5 μm per milliliter (a 1:20 dilution of a stock 0.1 M solution) is added. The suspension is shaken for 4 minutes. At the end of this period, the cells are repeatedly washed with saline and finally resuspended in saline to make a 25 per cent suspension. Any small clumps of cells that may be present can be easily broken up by repeated aspiration with a Pasteur pipette. The fact that the cells are properly coated is checked by Coombs' slide test with commercial serum. Our results have been reproducible with different batches of cells, chromic chloride, and gamma globulin. The slide test is then performed as an ordinary Coombs' slide test using the serum or euglobulin fraction to be tested. Two drops of 25 per cent cell suspension are mixed on a glass slide with two drops of the serum and the reaction is read over a light. The reactions are read as from 0 to 4 plus. (See Fig. 1.) Reactions of one plus or higher are considered positive. After the tests have been read, they may be allowed to dry in air at room temperature and saved. Reactions of 2 plus strength or greater are easily read in the dry state and negative readings remain negative. It is not possible, however, to distinguish 1 plus reactions from negative reactions in the dried state. These dried slides may be kept in slide boxes as permanent graphic records if desired.

RESULTS AND DISCUSSION

The results of using this test in clinical diagnosis are recorded in Table I. The diagnoses of rheumatic disease in the chart conform to the criteria proposed by Ropes and associates.[21]

TABLE I. RESULTS OF SLIDE TEST AND S.S.C.A. TEST IN 100 CASES

DIAGNOSIS	NO. OF CASES	SLIDE TEST		S.S.C.A. TEST	
		POSITIVE	NEGATIVE	1:64 OR>	<1:64
Definite rheumatoid arthritis	35	29	6	29	6
Probable rheumatoid arthritis	7	3	4	4	3
Possible rheumatoid arthritis	6	3	3	1	5
Degenerative joint disease	11	1	10	1	10
Gout	4	0	4	0	4
Disseminated lupus	2	0	2	0	2
Dermatomyositis	1	1	0	1	0
Late latent syphilis	1	0	1	1	0
Ankylosing spondylitis	2	1	1	1	1
Miscellaneous joint disorders: traumatic arthritis, shoulder hand syndrome, myofibrositis, bursitis, tenosynovitis, fascitis, peritendinitis, periarticular cellulitis, serum sickness	10	0	10	1	9
Erythema nodosum	2	1	1	0	2
Xanthomatosis	1	0	1	1	0
No organic disease	18	0	18	0	18

We found the slide test adaptable to the study of gamma globulin inhibition of rheumatoid factor activity and the precipitin reaction with gamma globulin since the cells were coated with the same gamma globulin used in the inhibition and precipitin studies, and the degree of inhibition could be assessed immediately by doing a slide test.

The data in Table II indicate that the titers of activity as determined by the slide test are of about the same order as in the S.S.C.A. test.* The lower

*We are unable to explain the 4 tube disparity between the S.S.C.A. titer and the slide test titer in the fourth example in Table II. Other sera tested since show either no disparity or, at most, a 2 tube disparity.

titers of the euglobulin fractions may represent the absence of the agglutination enhancing effect of serum.

TABLE II. SLIDE TEST WITH SERIAL DILUTIONS OF SERUM AND EUGLOBULIN

S.S.C.A. TITER (SERUM)	SLIDE TEST TITER (SERUM)	SLIDE TEST TITER (EUGLOBULIN)
1:1,024	1:512	1:16
1:256	1:256	1:16
1:1,024	1:512	1:32
1:1,024	1:64	1:8

The observations recorded in Tables I and III were made using serum. The results with euglobulin listed in Table II have already been mentioned. After these observations were made, studies on inhibition of the rheumatoid factor activity in the euglobulin fraction and corresponding formation of precipitate by the addition of gamma globulin were carried out. The data in Table IV are representative of our results. Before combining euglobulin fractions with gamma globulin solutions, the solutions were centrifuged at 8,000 r.p.m. for 15 minutes and the clear supernates used. This was done to insure clarity of the starting solutions, so that the formation of a precipitate could be detected early. We found that although maximum inhibition of rheumatoid factor activity as measured by slide test occurred within 15 minutes, maximum precipitation seemed to take at least 24 hours in most experiments.

TABLE III. SLIDE TEST OF POSITIVE SERUM WITH AND WITHOUT GAMMA GLOBULIN ADDED BEFORE TESTING*

	STRENGTH OF SLIDE TEST REACTION								
Serial dilutions	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Positive serum	4+	4+	4+	4+	4+	4+	4+	2+	2+
Positive serum plus equal volumes of 1% gamma globulin	4+	4+	3+	2+	1+	0	0	0	0

*Readings were done from coded tubes by two separate observers.

TABLE IV. CORRELATION OF RHEUMATOID FACTOR INHIBITION BY GAMMA GLOBULIN AND PRECIPITATE FORMATION*

TUBE NUMBERS	1	2	3	4	5	6	7	8	9	10	11
Positive euglobulin	All tubes contain 0.3 c.c. undiluted euglobulin										
Gamma globulin 0.3 c.c. 2.5%	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Slide test	0	0	0	0	0	+	1+	1+	2+	2+	2+
Precipitate	1+	1+	1+	1+	1+	2+	2+	0	0	0	0

*Readings were done from coded tubes by two separate observers.

In the later experiments, gamma globulin solutions to be combined with euglobulin fractions were heated at 60 to 65° C. for 15 minutes in order to obtain more rapid and clear-cut precipitin reactions. The results in Tables III and IV confirm the observations of others that rheumatoid factor is inhibited by prior mixing with gamma globulin. This inhibition correlated well with precipitin formation and presumably represents a combination of rheumatoid factor with some component of pooled gamma globulin.

SUMMARY

A modified Coombs' slide test has been described which compares favorably in diagnostic accuracy to other tests for rheumatoid factor activity. This test demonstrates the striking similarity of rheumatoid factor to rabbit anti-human globulin serum (Coombs' serum).

The chief advantages of this test are:

1. It can be performed and read within a few minutes once the reagents are prepared.

2. The end point is easy for anyone to read.

3. It can be done using the patient's serum or euglobulin fraction.

4. In the event that only occasional tests are done, the patient's own red cells may be used.

5. Dried slides may be filed as a permanent graphic record of test results.

6. It can be used in investigating rheumatoid factor since it can be done quickly at any step in processing rheumatoid factor to determine its presence or absence.

Using this test, observations of others regarding the combination of rheumatoid factor and gamma globulin have been confirmed.

The valuable assistance of Miss Virginia Castro is gratefully acknowledged.

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An Editorial

Advance

An *abnormal* serum reference standard has been announced.

The preparation of a *normal* serum reference standard for biochemical determinations was a step towards universal uniformity of laboratory tests. Further steps will facilitate discarding such time worn phrases as "normal value for this hospital", which have always implied that either the patients or methods have varied from hospital to hospital, and thus precluded the interchange of information between hospitals.

The next logical movement is the similar establishing of proven serum standards and controls in the abnormal or pathological range. No area of laboratory testing has needed such concentrated effort towards improvement, as that part concerned with determining the validity of abnormal results in blood chemistry. Until the present time, any result in the abnormal range had to be accepted in the wide range of abnormality. And an abnormal value, based on an aqueous or completely synthetic standard does not duplicate the physical and chemical problems of the patient's specimen, and cannot be considered the best basis for comparison in the diseased state. Among the most important of the interferences, not present in aqueous or synthetic protein-containing standards, are the lipids.

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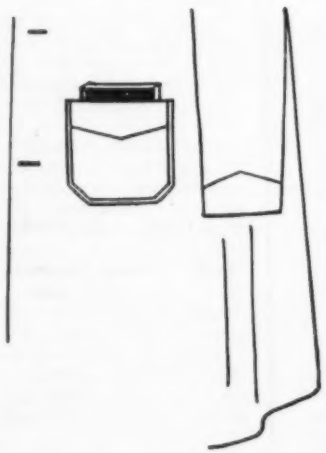

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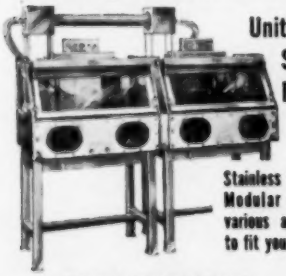

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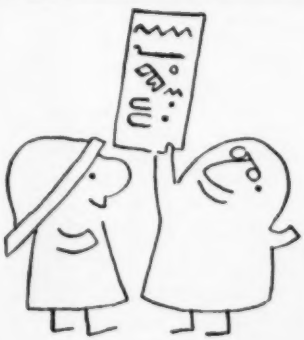

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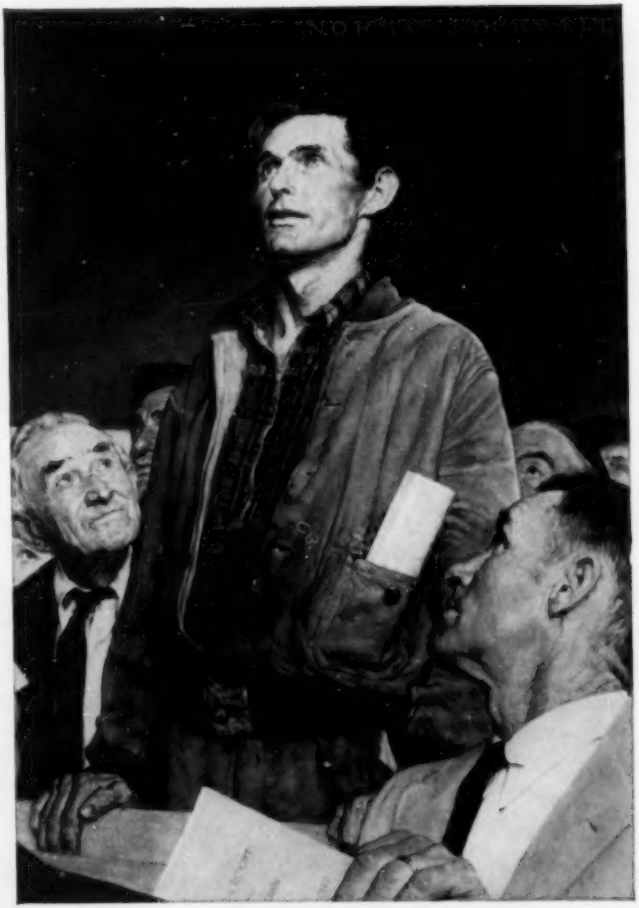

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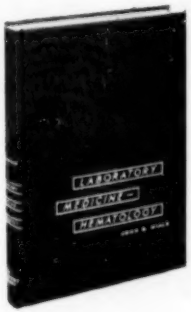

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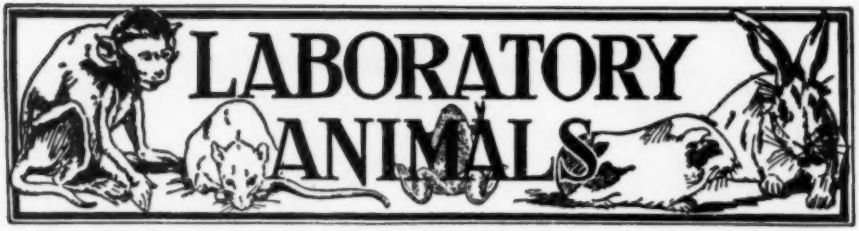

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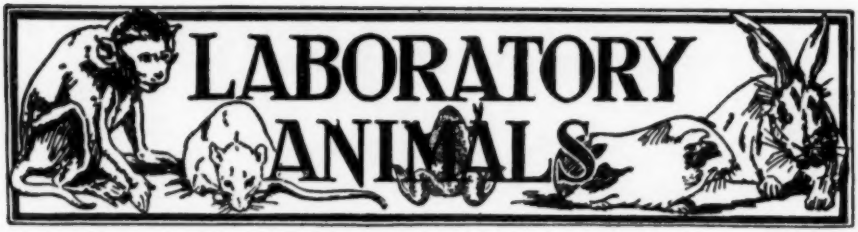

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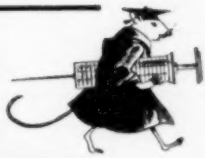

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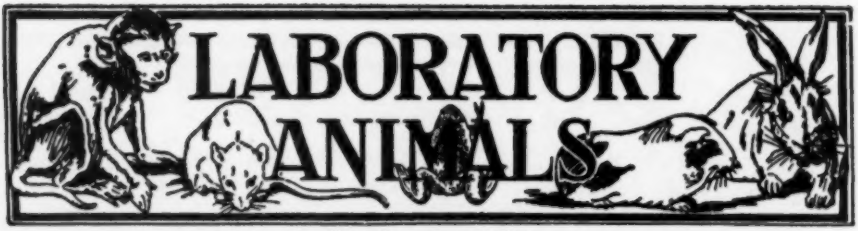

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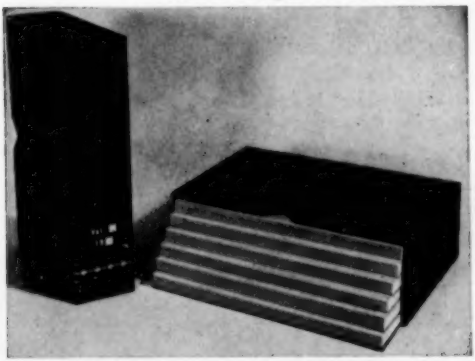

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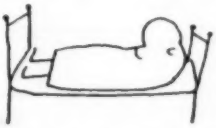

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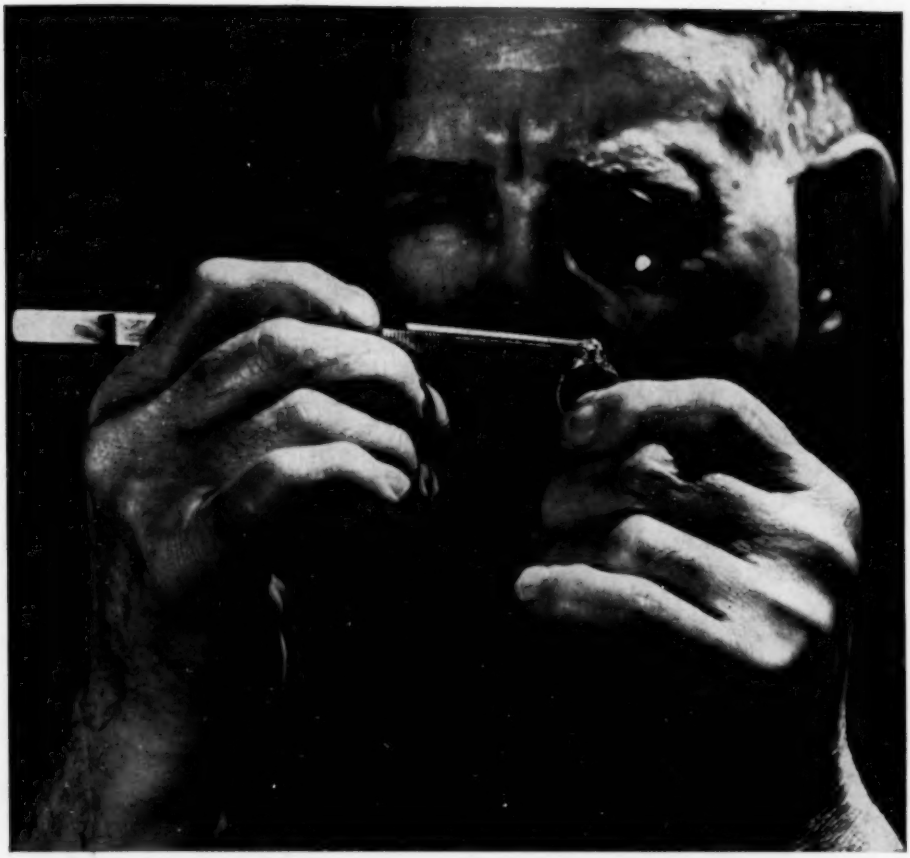

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